TESE DE DOUTORAMENTO

EVALUATION OF THE INTRA AND INTERSPECIFIC VARIABILITY IN THE GENUS *Perkinsus*. PROTEOMIC ANALYSIS OF THE PARASITE AND ITS INTERACTION WITH THE HOST

Asdo. .....

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SANTIAGO DE COMPOSTELA 2015

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CENTRO DE INVESTIGACIÓNS MARIÑAS (XUNTA DE GALICIA)

UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

Evaluación de la variabilidad intra e interespecífica en el género *Perkinsus*. Análisis proteómico del parásito y de su interacción con el hospedador.

Memoria presentada por

SERGIO FERNÁNDEZ BOO

para optar al título de doctor en Biología

Codirectores

Antonio Villalba García

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Santiago de Compostela, Enero 2015

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INFORMAN:

De que la memoria titulada "Evaluation of the intra and interspecific variability in the genus *Perkinsus*. Proteomic analysis of the parasite and its interaction with the host", que presenta D. Sergio Fernández Boo para optar al grado de Doctor por la Universidad de Santiago de Compostela, ha sido realizada bajo su dirección y considerándola concluida, autorizan su presentación a fin de que pueda ser juzgada por el tribunal correspondiente.

Y para que así conste, se firma el presente informe

Fdo. Dr. Antonio Villalba García. Fdo. Dra. María Asunción Cao Hermida

Fdo. Dr. Paulino Martínez Portela.

Santiago de Compostela, a 30 de Enero de 2015

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D. Carlos Pereira Dopazo, Doctor en Biología y Profesor Titular del Dpto. de Microbiología y Parasitología de la Universidad de Santiago de Compostela,

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Fdo.: Dr. Carlos Pereira Dopazo

Santiago de Compostela, a 30 de Enero de 2015.

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Durante el desarrollo del presente trabajo Sergio Fernández Boo ha disfrutado de una beca de formación de personal investigador de la Xunta de Galicia (DOGA nº11, 16 de Enero de 2008, 1058-1065).

La presente tesis doctoral ha sido desarrollada en el Centro de Investigacións Mariñas (CIMA), perteneciente a la Xunta de Galicia, a través de los proyectos siguientes:

- Proyecto: "La perkinsosis en el litoral español: caracterización de variantes taxonómicas del parásito, de su ciclo de vida y de la respuesta inmunitaria del hospedador". Duración: 01/10/2006-31/12/2009 (3 años). Financiado por el Ministerio de Ciencia e Innovación. Nº Ref: AGL2006-11809/ACU.

- Proyecto: "Análisis proteómico de la interacción del sistema inmune de la almeja con *Perkinsus olseni*". Duración: 14/12/2009- 13/12/2010 (1 año). Financiado por el Ministerio de Ciencia e Innovación. Nº Ref: AGL2009-13305C03-01.

- Proyecto: "Perkinsosis de la almeja: expresión proteínica en los hemocitos y plasma del hospedador durante su interacción". Duración: 01/01/2012-31/12/2014 (2 años). Financiado por el Ministerio de Ciencia e Innovación. Nº Ref: AGL2011-30449-C02-01.

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AGRADECIMIENTOS

Me gustaría agradecer a todos las personas que me han ayudado a que esta tesis haya salido adelante.

En primer lugar me gustaría agradecer a mis directores de tesis, Antonio Villalba, Asun Cao y Paulino Martínez porque sin vuestros consejos y vuestra ayuda esto no podría ser una realidad.

Agradecer a Christopher Dungan y a Jerome La Peyre que gentilmente nos facilitaron los cultivos de *Perkinsus chesapeaki, Perkinsus olseni* procedentes de Japón y Nueva Zelanda y *Perkinsus marinus* respectivamente.

Gracias a la gente del grupo de patología: Maribel Meléndez, Elena Penas, Susi Carballal, Laura Martín por acogerme tan bien y por el gran ambiente que se respira en él. A todo el personal de CIMA que sin duda han ayudado a que estos cuatro años hayan sido más que buenos.

Quiero agradecer también a mis dos grandes amigas y compañeras, Andrea Ramilo y Lucía Barreiro que con sus risas y sus confidencias han hecho que los días pasasen más rápido.

A Viki que sin su ayuda no hubiese sido posible hacer tanto en tan poco tiempo.

A Rocío Vicente, José Manuel Ramos, Mariano Marletta y Mar González que han ayudado a dar soporte técnico a lo largo de estos cuatro años.

A Eduardo Chicano que me ha enseñado mucho sobre la proteómica y me he reído tanto con sus bromas. No me olvido tampoco de Alba Ruíz, Laura Medina y Tina Domínguez que tan bien me lo han hecho pasar en Córdoba.

A Manuela Saavedra, Silvia Alcalde, Maika Rodríguez y Yova López que me aguantan en los cafés en la ofi.

Y a toda mi familia por el apoyo recibido.

Muchas gracias.

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Abbreviations used in the dissertation

2-DE – Two Dimensional Electrohoresis

A

AFLP – Amplified Fragment Length Polymorphism

AMPs – Antimicrobial Peptides

AP – Acid Phosphatase

APX – Ascorbate Dependent Peroxidase

ATP – Adenosin Triphosphate

B

BLAST – Basic Local Alignment Search Tool

BRD – Brown Ring Disease

C

cDNA – complementary Deoxyribonucleic Acid synthesized from a messenger ribonucleic acid

CHAPS - 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

CID – Collision Induced Dissociation

COX – Citochrome Oxidase Complex

CvSI – *Crassostrea virginica* Serine Protease Inhibitor

D

DDE - p,p´-dichlorodiphenyldichloroethylene

DIGE - Differential Gel Electrophoresis

DNA – Deoxirybonucleic Acid

DTT – Dithiothreitol

E

ECD – Electron Capture Dissociation

ECPs – Extracellular Products

EDTA – Ethylenediaminetetraacetic acid

ESI – Electrospray Ionisation

EST – Expressed Sequence Tags

ETD – Electron Transfer Dissociation

F

FAO – Food and Agriculture Organization

FASTA – Fast Alignment

FTM – Fluid Thioglycollate Medium

G

GO – Gene Onthology

GSH – Glutathione

GST – Glutathine S-Transferase

GTP – Guanosine Triphosphate

H

H_2O_2 – Hydrogen peroxide

HIF – Hypoxia inducible factors

HOCl – Hypoclorite

HSP – Heat Shock Protein

I

ICAT – Isotope-coded Affinity Tag

ICR – Ion Cyclotron Resonance

IEF – Isoelectrical Focusing

IGS – Intergenic spacer

IPG – Immobilised pH Gradient

iTRAQ – isobaric Tags for Relative and Absolute Quantification

ITS – Internal Transcribed Spacer

K

kDa – kilo Daltons

L

LAMP – Loop-mediathed Isothermal Amplification

LC-MS/MS – Liquid Chromatography coupled to Mass Spectrometry

LPS – Lipopolysaccharide

LSU – Large SubUnit

M

MALDI – Matrix-Assisted Laser Desorption and Ionisation

MCL – Manila Clam Lectin

MLGs – Multilocus Genotypes

MPO – Mieloperoxidase

MS – Mass Spectrometry

Mw – Molecular weight

N

NCBI – National Center for Biotechnology Information

NNP - Nicotinate-Nucleotide Pyrophosphorylase

NO – Nitric Oxide

Nramp – Natural Resistance-Associated Macrophage Protein

NTS – Nontranscriber Spacer

O

O^{-}_{2} – Superoxide anion

OIE – World Organization for Animal Health

$ONOO^{-}$ - Peroxinitrite

P

PAGE – Polyacrylamide Gel Electrophoresis

PAMPs – Pathogen Associated Molecular Patterns

PCR – Polymerase Chain Reaction

PFF – Peptide Fragment Fingerprinting

pI – Isoelectric Point

PMF – Peptide Mass Fingerprinting

PO – Phenoloxidase

PRX – Peroxiredoxin

PS – Phosphatidylserine

PSAT - Phosphoserine Aminotransferase

Q

QTLs – Quantitative Trait Locus

R

RACK – Receptor of Activated C Kinase

RAPD – Randomly Amplified Polymorphic DNA

RFLP – Restriction Fragment Length Polymorphism

RNA – Ribonucleic Acid

ROIs – Reactive Oxygen Intermediates

ROS – Reactive Oxygen Species

S

SABL – Sialic Acid Binding Lectin

SCP – Sarcoplasmic Calcium Binding Protein

SDS – Sodium Dodecyl Sulphate

SILAC – Stable Isotopic Labelling by Amino Acid in Culture

SL – Spliced Leades

SOD – Superoxide Dismutase

SRP - Signal Recognition Particle

SRβ - Signal Recognition Particle Receptor Subunit β

SSH – Supression Sustractive Hybridization

SSR – Single Sequence Repeats

SSU rRNA – Small Subunit of the Ribosomal Ribonucleic Acid

STR – Short Tandem Repeats

T

TOF – Time of flight

U

UV - Ultraviolet

USA – United States of America

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Index

I. INTRODUCTION

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I.1. PRODUCTION OF CLAMS

Aquaculture of clams is an industry that is rising every year. A perspective of the world clam production in the last ten years shows an increment of the production of 74,5%. The clam species with the highest worldwide production is the Manila clam *Ruditapes philippinarum*. China is the main producer of this species, followed by Italy, South Korea, USA and Spain. The production of this species reached 3,785,311 tonnes in 2012. (FAO Fisheries and Aquaculture Statistics. http://www.fao.org/ fishery/ statistics/ en) (Fig. I.1).

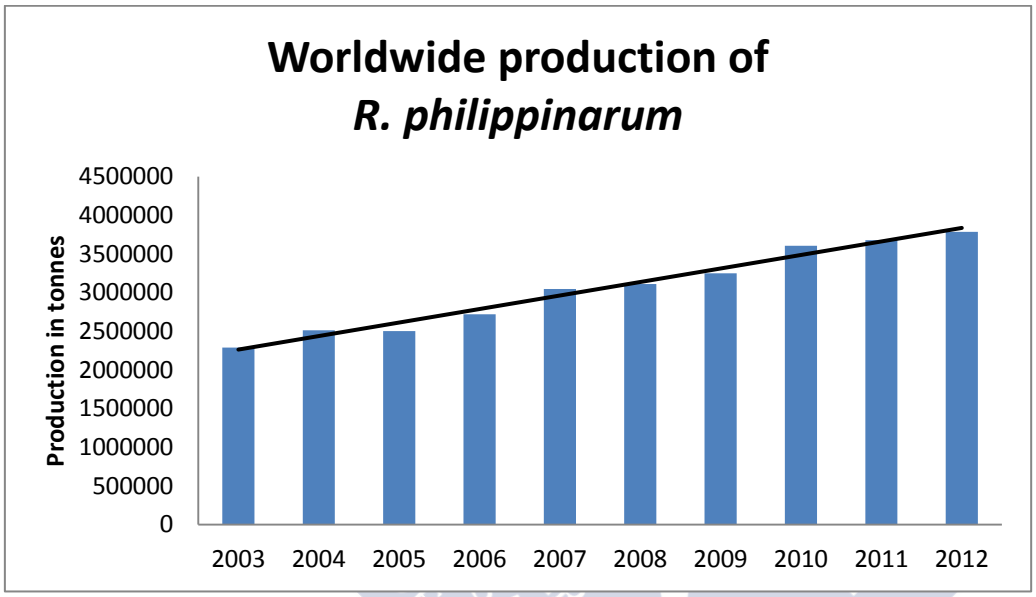

Fig. I.1. World clam production of the last 10 years. FAO Fisheries and Aquaculture Statistics.

The production of venerid clams in Galicia, based both on fishery and aquaculture, is a very important socio-economic resource, directly employing 4000 people, most of them women. In some coastal populations, venerid clam industry is the main source of incomes. This activity generated about 67,6 m€ in the year 2013, with a production of 9,832 tonnes (www.pescadegalicia.com). The introduction of foreign species in marine ecosystems is an established method to increase productivity and generate incomes. The introduction of the Manila clam *Ruditapes philippinarum* in Europe during the 1970s and 80s is a good example (Gosling, 2002). Reproduction of Manila clam along the European coastline resulted in its rapid spread and naturalisation. *R. philippinarum* has proved to be well adapted and faster growing than the native European grooved carpet shell clam *Ruditapes decussatus* (Jensen et al., 2004; Hurtado et al., 2011). Consequently, *R. philippinarum* is the major contributor to clam landings in Europe. In Galicia, *R. philippinarum* culture rose the last years (Fig. I.2) being the clam species with the highest culture production in 2013 (1,350 tons), followed by *Venerupis corrugata* (273,5 tons) and *R. decussatus* (184,2 tons) (www.pescadegalicia.com).

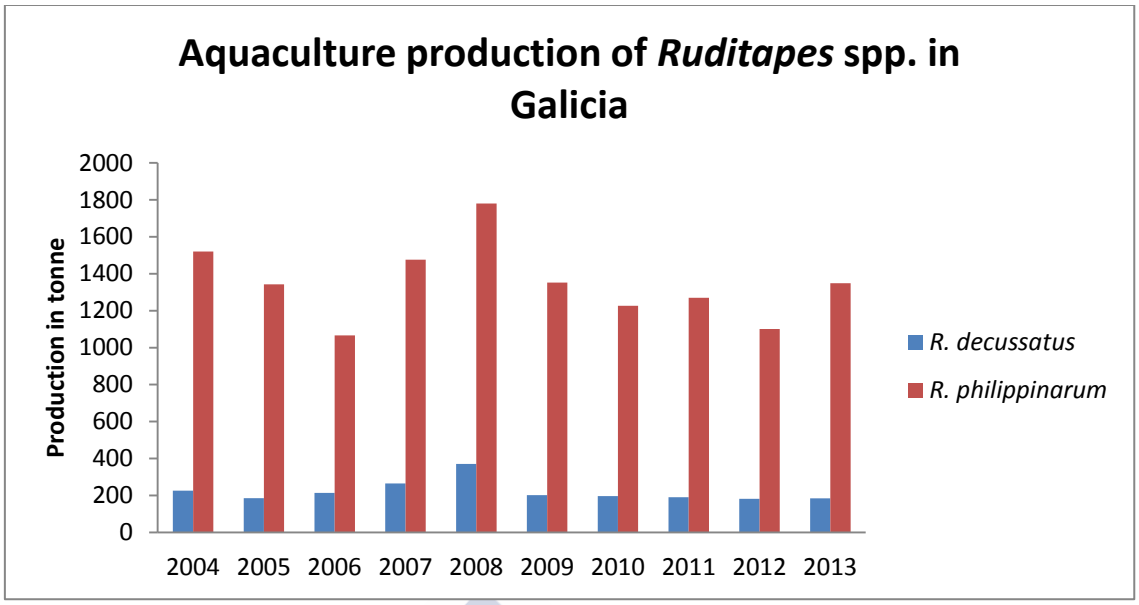

Fig. I.2. Evolution of the annual aquaculture production of *Ruditapes decussatus* and *Ruditapes philippinarum* in Galicia for the period 2004-2013. www.pescadegalicia.com.

Additionally, venerid clam production from fisheries Galicia is even higher (Fig. I.3). The fishery production of *R. decussatus* has remained constant in the last 13 years while the production of *R. philippinarum* shows an increasing trend since 2001. Nowadays, *R. philippinarum* is the venerid clam species with the highest production in Galicia.

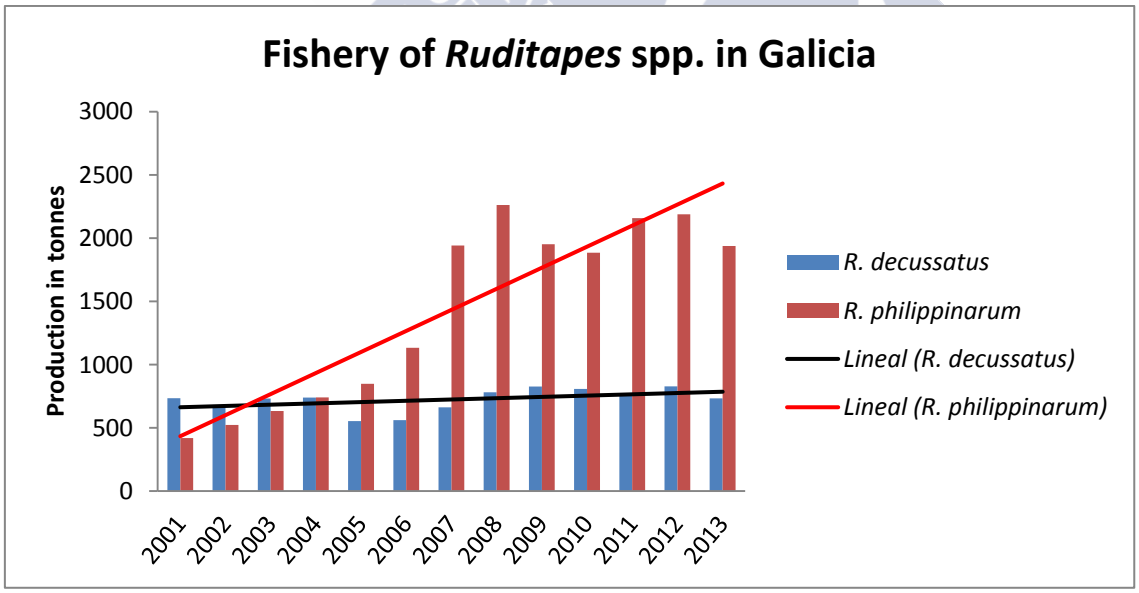

Fig. I.3. Evolution of annual fishery production (columns) of *Ruditapes decussatus* and *Ruditapes philippinarum* in Galicia for the period 2001-2013. The lines correspond to linear adjustment. www.pescadegalicia.com

I.1.1. Pathological conditions of clams R. philippinarum *and* R. decussatus*.*

The research in mollusc pathology progresses as mollusc aquaculture spreads. The main pathological problems of *R. decussatus* and *R. philippinarum* are summarised in Table I.1.

Table I.1. Main pathogens of the clams *Ruditapes decussatus* and *Ruditapes philipinarum*

Species	Herpes-like virus	
	Host type:	*R. philippinarum*
	Mortality:	Yes (larvae)
	Reference:	Renault and Arzul, 2001.
Species	Picornavirus	
	Host type:	*R. decussatus*
	Mortality:	Yes
	Reference:	Novoa and Figueras, 2000.
Species	Rikettsia-like bacteria	
	Host type:	*R. decussatus*
	Mortality:	Not reported
	Reference:	Navas et al.,1992; Villalba et al., 1993; López et al., 1998.
Species	*Vibrio tapetis*	
	Host type:	*R. philippinarum and R. decussatus*
	Mortality:	Yes
	Reference:	Paillard and Maes, 1994; Borrego et al., 1996; Drummond et al., 2007.
Species	*Minchinia tapetis*	
	Host type:	*R. decussatus and R. phillipinarum*
	Mortality:	Not reported
	Reference:	Joly, 1982; Chagot et al., 1987; Villalba and Navas, 1988; López et al., 1998
Species	*Perkinsus olseni*	
	Host type:	*R. philippinarum and R. decussatus*
	Mortality:	Yes
	Reference:	Azevedo, 1989; Santmarti et al., 1995, Villalba et al., 2005; Pretto et al., 2014
Spcies	*Cercaria tapidis*	
	Host type:	*R. philippinarum*
	Mortality:	Not reported
	Reference:	Lee et al., 2001.
Spcies	*Cercaria lata/Bacciger bacciger*	
	Host type:	*R. decussatus*
	Mortality:	?
	Reference:	Breber, 1985; Culurgioni et al., 2006; El-Wazzan and Radwan, 2013

The introduction of foreign species for aquaculture is frequently associated with spreading of new diseases. That could be the case of two diseases with devastating potential, perkinsosis and brown ring disease (BRD), which have been reported in venerid clams in Europe associated with the introduction of the Manila clam *R. philippinarum*.

Vibrio tapetis, the causative agent of BRD, constitutes a major limiting factor for the cultivation of Manila clams (*Ruditapes philippinarum*) in Europe. This bacterium was first isolated in Landela (France) in 1990 and was designated *Vibrio* P1 (VP1) (Paillard and Maes, 1994), and later described as the new species *Vibrio tapetis* (Borrego et al., 1996). *V. tapetis* colonises the surface of the epithelia of the mantle and the periostracal lamina, causing a disruption of the normal calcification process (Allam et al., 2014). This alteration provokes the progressive deposition and accumulations of the periostracal lamina on the inner surface of the shell producing the typical brown conchiolin deposit within the extrapallial space (Paillard and Maes, 1994; Allam et al., 2014). Evidence suggests that the bacteria can penetrate into the mantle through lesions in the epithelium of heavily infected clams, subsequently spreading throughout the body to cause severe systemic infection and death (Allam et al., 2002).

The infection with *Vibrio tapetis* and that with *Perkinsus olseni* are the two most serious diseases for the culture of *R. philippinarum* and *R. decussatus* because of both pathogens disseminate quickly and can be lethal. *Perkinsosis* in European clams was detected for first time in Italy (Da Ros and Canzonier, 1985). Episodes of mortality of *R. decussatus* in Southern Portugal evidenced the importance of perkinsosis (Ruano and Cachola, 1986; Azevedo, 1989). Mortalities reaching up to 100% were reported in *R. philippinarum* from Catalonia (Santmartí et al., 1995). In Galicia the detection of Perkinsus –like parasites in *R. decussatus* in the late 1980´s (Figueras et al., 1992) was considered a threat to the clam industry of the region (Villalba et al., 2005). Annual cumulative mortality in a Galician clam bed was estimated over 40 % by Villalba et al. (2005) in a five years study. *Perkinsus olseni* was also blamed for mass mortalities in Italy and Korea where perkinsosis was associated with an 80%-100% decrease in *R. philippinarum* landings (Park and Choi, 2001; Pretto et al., 2014). More information on perkinsosis is provided in the section 3.

I.2. IMMUNE SYSTEM OF *R. philippinarum* AND *R. decussatus*

Immunity is defined as a reaction against live or inert exogenous substances, including microorganisms and macromolecules as proteins or lipopolysaccharides (Abbas and Lichtman, 2000). The immune system of molluscs consists only of innate immunity. External barriers such as shells, mucus and epithelia constitute the first line of defense against pathogens and parasites; when these barriers are breached, the

second, internal line of defense involving cellular and soluble (humoral) haemolymph components come into play (Amstrong, 2006; Binias et al., 2014). While the innate immune system is often regarded as an evolutionary more ancient and hence more primitive form of immunity than the adaptive responses seen in vertebrates, it is actually a surprisingly complex and efficient form of protection against many parasites and pathogens encountered by molluscs (Sokolova, 2009). Innate immune system is able to recognise specific molecular patterns associated to pathogens called pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 2000; Soudant et al., 2013).

I.2.1. Haemocytes

Clams, as other bivalves, have developed an open, haemolymphatic circulatory system. Haemocytes are circulating cells which act as sentry cells, scanning the immediate environment to detect foreign material and induce efficient immune responses (Donaghy et al., 2009). The health status of marine bivalves can be inferred from immune parameters, such as haemocyte concentration and phagocytosis following natural or experimental contact with pathogens (Allam et al., 2006; Binias et al., 2014). In addition, humoral factors released by haemocytes or other cell types may help to fight against invasive microorganisms. Haemocytes are involved in different function as maintenance bivalve biology and physiology, tissue and shell formation, maintenance of homeostasis, nutrition, excretion, wound healing, and immune response. Two main haemocyte types were described according to morphological features: granulocytes, which contain numerous granules within the cytoplasm, and agranulocytes also called hyalinocytes, without granules. The granulocyte subset is homogeneus, whereas subpopulations of agranulocytes were observed in some but not all bivalve species as small and large hyalinocytes (Donaghy et al., 2009). These two types of haemocytes were found in *R. decussatus* and *R. phil*ippinarum clams but a more specific classification attending on the basis of different dye affinities of cytoplasmatic granules was developed. The haemocytes of *R. philippinarum* were classified into three categories by light microscopy: basophils, acidophils and neutrophils (López et al., 1997b, Cima et al., 2000). Cheng (1981) suggested that the occurrence of various types of granules might be related to differentiation and maturation process; specifically, basophilic granules were hypothesised to be immature granules which mature and become acidophilic. The simultaneous occurrence of both kinds of granules within some haemocytes in *R. decussatus* and *R. philippinarum* might support this hypothesis (López et al., 1997b). Although some authors reported differences in size between granulocytes and hyalinocytes, these two cell types seem to possess similar ranges of diameter approximately 10 µm (Donaghy et al., 2009).

I.2.2. Functions of haemocytes

Haemocyte sub-populations may be differentially involved in functions of bivalve biology and physiology, including nutrient transport and digestion, tissue and shell formation, maintenance of homeostasis, and immune response (Cheng, 1996). In bivalves, cellular defense mechanisms against foreign biological material can be summarised in five steps: 1. Chemotaxis; 2. Opsonisation; 3. Recognition of non-self particles; 4. Phagocytosis; 5. Intracellular degradation of foreign material (Donaghy et al., 2009).

I.2.2.1. Chemotaxis

Migration of the haemocytes through the haemolymph or tissues addressed to meet the foreign material is called chemotaxis. The active migration is induced and regulated by soluble molecules named chemotaxins, released either by the foreign agent or by the host cells. Phagocytic and chemotactic activities of haemocytes from *R. decussatus* and *R. philippinarum* have been studied using bacterial challenge with *Vibrio tapetis*, the causative agent of brown ring disease. All the extracellular products of the bacterial strains contain chemoattractans to haemocytes (López-Cortes et al., 1999). The occurrence of milky-white nodules on the surface of mantle, gill and foot tissue is a common feature of *Perkinsus*-infected *R.philippinarum*; this nodules are formed by inflammation as a consequence of a massive haemocyte infiltration into tissues, surrounding *Perkinsus* trophozoites, suggesting active migration of haemocytes from haemolymph into tissues (Park and Choi, 2001). Similarly, exposure of Manila clams to the harmful alga *Prorocentrum minimum* induced inflammatory response, characterised by massive haemocyte incursion into digestive glands, intestine and stomach (Hégaret et al., 2009).

I.2.2.2. Secretion of soluble factors (humoral factors)

Various constitutive or inducible humoral defense factors including lectins, antimicrobial peptides, lysosomal enzymes, major plasma proteins, protease inhibitors, and cytokine-like molecules have been described in bivalves (Soudant et al., 2013). Many of these molecules are released by haemocytes.

- *Lectins*

Lectins are soluble or membrane-found factors that recognise specifically, and bind reversibly to, the carbohydrate-containing molecules of foreign cells. Lectins play agglutinating and opsonising roles, facilitating interaction of the surface of haemocytes with foreign particles (Ordás et al., 2000). Several studies have demonstrated that the infection by external agents such as *Perkinsus olseni* or bacteria as *Vibrio* spp. induces the production of different types of lectins in clams (Bulgakov et al., 2004; Kang et al., 2006; Kim et al., 2008a, 2008b; Moreira et al., 2012). Bulgakov et al. (2004) found a

Manila clam lectin (MCL) with carbohydrate specificity for *N*-acetyl-D-galactosamine, which contains both mucin-type carbohydrate chains and highly branched mannans (plant polysaccharide polymer of mannose). This lectin was probed to bind at the surface of hypnospores from *Perkinsus* sp. Lectins could be specific to an infection; Kang et al. (2006) reported that lectins expressed in *P. olseni* infection were different from the lectin expression for *Vibrio tapetis* infection. Fluorescent beads coated with purified C-type lectin from Manila clam were actively phagocytosed by haemocytes from the clam, proving that lectins operate as opsonin through recognition of terminal GalNAc/Gal residues on the parasite (Kim et al., 2006; Kim et al., 2009). A novel isoform of the MCL, designated MCL-4, was isolated from plasma of *R. philippinarum* and showed great opsonisation ability against *Vibrio tubiashi* cells (Takahashi et al., 2008). Another type of lectins is the sialic acid-specific lectins, these lectins were also detected in Manila clams (MCsialec) infected with *P. olseni*. MCsialec was detected in each tissue of the clams and upon infection with *P. olseni* or *V. tapetis* (Adhya et al., 2010). A new C-type lectin gene was identified by Mu et al. (2014) in *Ruditapes philippinarum* showing the great variety of different lectins and recognition molecules involved in the innate immune system of the clams.

- *Antimicrobial peptides*

Antimicrobial peptides (AMPs) are characterised by their small size (12-100 amino acids), heat stability, and cationic and hydrophobic nature. Their pore forming action, and/or detergent effect, disrupts the microorganisms membrane and causes cellular lysis, thus playing a role in host defense against microbial invasion (Li et al., 2009). Several marine bivalves have been reported to produced AMPs, these peptides are generally produced and stored in haemocytes to be released upon pathogen invasion. A polypeptide of *R. philippinarum* and *R. decussatus* against *P. olseni* infection has been reported; this polypeptide is heavier than other AMPs (225 kDa) (Montes et al., 1995b) and it is specific of *Perkinsus* infection because it is not released against other microorganism infections (Montes et al., 1996). Kang et al. (2006) described a clam AMP similar to mussel defensin in Manila clams infected with *P. olseni*. Two more antimicrobial peptides with similarity with myticin and mytilin AMP previously identified in *Mytilus galloprovincialis* (Mitta et al., 1999) were identified in clams *R. philippinarum* (Gestal et al., 2007). Clam mytilin is closer to Mytilin isoform C. It is accepted that these myticins are essentially active against gram-positive bacteria, and less active against gram-negative bacteria and fungi in mussels (Mitta et al., 2000). The variation in aminoacid residues of the different AMP types in each species could generate a specific differentiation for recognition of different types of pathogens (Gestal et al., 2007).

- *Lysozyme*

Lysozyme plays a role in both defense and digestion in bivalves. Bacteria also constitute a portion of a bivalve´s diet and lysozyme can break down cell wall due to its muramidase activity (Soudant et al., 2013). The effect of perkinsosis in the lysozyme activity is still unclear, some works detect no significant effect in lysozyme concentration either in *C. virginica* infected with *P. marinus* (Chu and La Peyre, 1989; Chu and La Peyre, 1993) or in *R. decussatus* infected with *P. olseni* (Ordás et al., 2000), while a light diminution was detected in *C. virginica* serum by La Peyre et al. (1995a).

- *Heat shock proteins (HSP)*

Cell exposition to high temperatures induces the synthesis of some proteins called heat shock proteins (HSP). The expression of these proteins could be due to several reasons such as hypoxia, presence of heavy metals, physical or chemical agents and every factor which produce a cellular stress. Heat shock proteins are molecular chaperones that protect the cell and maintain homeostasis under stressful conditions (Cellura et al., 2007; Gestal et al., 2007). Although HSP have been utilised as molecular marker of stress factors, the modulation of these proteins have been also related to immune process. The up-regulation of HSP 70 gene in cDNA libraries of haemocytes from *Ruditapes decussatus* challenged with bacteria (Gestal et al., 2007), and *R. philippinarum* challenged with *P. olseni* (Kang et al., 2006) support the hypothesis that HSP could have an important role in immune response.

- *Prophenol oxidase system (proPO)*

The synthesis of melanin to participate in the encapsulation immune response is modulated by phenoloxidase (PO). After stimulation by invading pathogens, proPO is activated to become PO, which is the responsible for initiating the formation of melanin (Johnson et al., 2003; Cong et al., 2005). The activation of this enzyme could be modulated by β-glucans from yeast and bacteria cell wall, lipopolysaccharides and peptidoglycans (Costa et al., 2008). The step from proPO to active PO is modulated by complex enzyme cascade of serine proteases. Despite PO pathway is a well characterised system in insects, very few reports are available in molluscs and only one report was developed in clams describing for first time this immune pathway in *R. philippinarum* (Cong et al., 2005).

- *Major plasma proteins*

Major plasma proteins are a group of proteins sharing high sequence homology in some marine bivalve molluscs, and it is thought to play a role in host defense. A major plasma protein of the eastern oyster, named dominin, has been purified and fully characterised (Itoh et al., 2011). The amino acid sequence of dominin was

reported to be similar to the major plasma proteins cavortin of the Pacific oyster (Scotti et al., 2007), pernin of the green-lipped mussel, *Perna canaliculus* (Scotti et al., 2001), and a protein labelled as an extracellular superoxide dismutase from the Sydney rock oyster *Saccostrea glomerata* (Green et al., 2009). Dominin and its homologs are believed to be multifunctional proteins. They are possibly involved not only in host defense, but also antioxidation, wound repair, metal transportation and mineralisation. Although expression of dominin gene was up regulated following *P. marinus* challenge, its defense role remains uncertain (Itoh et al., 2011). No major plasma proteins have been isolated from *Ruditapes* spp. but other clam species, such as *Mya arenaria* or *Mercenaria mercenaria*, show similar proteins (Abebe et al., 2007).

- *Proteases and inhibitors of proteases*

Protease proteins are present in pathogen organisms and are able to neutralise defence mechanisms of the host. These proteases facilitate the invasion of the pathogen into the host tissues and can reduce the lysozyme activity and haemocyte agglutination titre (La Peyre et al., 1996). Several organisms developed inhibiting enzymes to block the activity of pathogen proteases, theses enzymes were called protease inhibitors or antiproteases. Serine protease inhibitors also exist in eastern oysters and were proposed to be involved in the elimination of *P. marinus* (Faisal et al., 1998, Oliver et al., 2000). Two serine protease inhibitors, CvSI-1 and CvSI-2, have been purified and characterised from eastern oyster plasma (Xue et al., 2006; Xue et al., 2009; Yu et al., 2011). The ability of CvSI-1 to inhibit *P.marinus* proliferation *in vitro* was confirmed (La Peyre et al., 2010). Serine proteases inhibitors have not been described in clams yet but it is reasonable to consider that clams utilise these kinds of inhibitors to fight against pathogens as well as other bivalves.

I.2.2.3. Encapsulation

Encapsulation was proposed as an aborted phagocytosis when the target is too large to be phagocytosed (Cheng and Rifkin, 1970). A series of concentric layers formed by fibroblasts, mucupolysaccharides and glycoproteins surrounding the target foreign particle is built by haemocytes (Cheng and Rifkin, 1970). In clams infected with *P. olseni*, milky white nodules can be observed with naked eye in the clam body surface. These nodules correspond to encapsulation of parasite trophozoites, which are surrounded by a capsule mainly composed of eosinophilic, granular haemocytes (Fig. I.4) (Park and Choi, 2001). Indeed, observation of phagocytosis of *P. olseni* by clam heamocytes in histological sections is much less frequent than in the eastern oyster infected by *P. marinus* (Casas et al., 2002a). After encapsulation, it is believed that lysosomal enzymes from granulocytes, likely acting together with ROS production, destroy the encapsulated parasites (Soudant et al., 2013). Although is unclear how efficiently *Perkinsus* spp. cells can be degraded and/or eliminated, this encapsulation

process could block trophozoite dissemination via the circulatory system (Montes et al., 1995a).

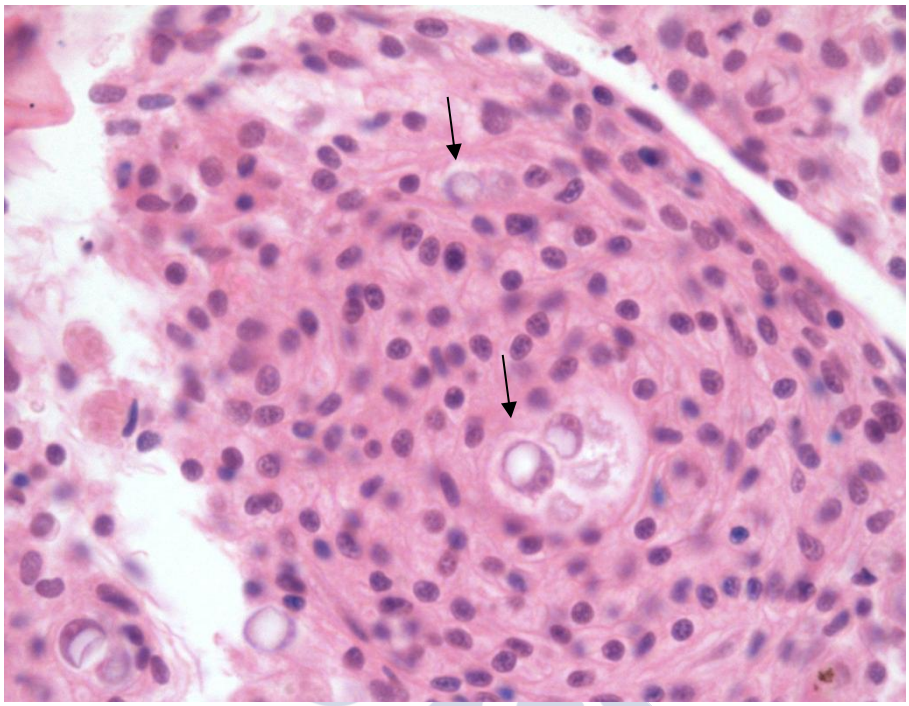

Fig. I.4. Image of a histological section of a clam *Ruditapes decussatus* showing encapsulation of *Perkinsus olseni* (arrows) by layers of haemocytes.

I.2.2.4. Phagocytosis

The major cellular mechanism involved in the hemocyte mediated immune response of marine bivalves is the internalisation of foreign material. Recognition and binding of non-self material by receptors expressed at the surface of the haemocytes induce the mechanism of internalisation called phagocytosis (Fig. I.5) (Donaghy et al., 2009). The attraction of haemocytes to foreign particles is mediated by chemotaxis and the recognition of foreign particles is produced by interaction of haemocytes and PAMPs. In clams, engulfment of foreign particles by haemocytes occurs by invagination of the cell membrane followed by pseudopod formation and particle internalisation into an endocytic vacuole, also called the primary phagosome. Cytoplasmic lysosomal granules then migrate and fuse with the primary phagosome. Contents of granules with numerous hydrolases, including phosphatases, esterases, amidases, as well as carbohydrate hydrolases, and oxidative enzymes such as peroxidase and cytochrome c oxidase (López et al., 1997a; Cima et al., 2000; Donaghy et al., 2009), are subsequently discharged in the so-called secondary phagosome, accomplishing the enzymatic degradation of engulfed foreign material. After the intracellular destruction of a pathogen, granules of glycogen are released that could be utilised in haemocytes metabolic processes or liberated to plasma (Costa, 2008).

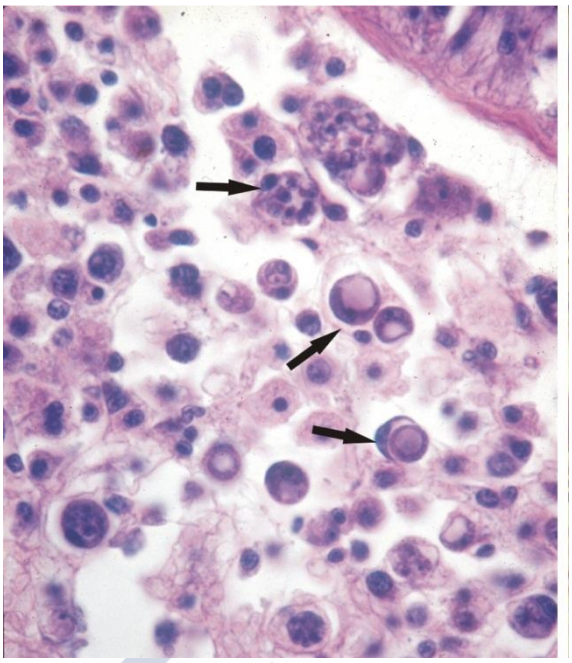

Fig. I.5. Immune response of the oyster *Crassostrea virginica* against *Perkinsus marinus*. Histological section of an oyster showing oyster haemocytes (arrows) that have phagocytosed *P. marinus*. Image taken from Villalba et al. (2011).

I.2.2.5. Oxidative mechanisms

A high proportion of reactive oxygen species (ROS) are generated during phagocytosis and this process is called "respiratory burst". The respiratory burst is generally described as a cascade of enzymatic reactions that starts with the production of the superoxide anion (O_2^-) by NADPH oxydase complexes associated with the plasma membrane (Schott and Vasta, 2003; Donaghy et al., 2009). Superoxide is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), and in presence of chloride ion, H_2O_2 is the substrate for the production of hypochlorite (HOCl), in a reaction catalysed by myeloperoxidase (MPO) (Anderson et al., 1992; Greger et al., 1995). Cytotoxic effects of ROS include peroxidation of lipids, breakage of DNA strands, and inactivation of enzymes, especially those containing Fe-S centers (Schott and Vasta, 2003).

ROS production is inhibited in *C. virginica* haemocytes when are exposed to live *P. marinus* trophozoites (La Peyre et al., 1995a; Volety and Chu, 1995; Anderson, 1999; Schott and Vasta, 2003). This inhibition allow to parasite to be alive inside oyster haemocytes. Haemocytes of *R. philippinarum* can also produce ROS upon their activation (Cima et al., 2000) but exposure to *P. olseni* seems to have no significant effect in the production of ROS by clams haemocytes (Hégaret et al., 2007; da Silva et al., 2008) as well as exposure to β-glucans (Costa et al., 2008). Among ROS produced in the respiratory burst, the superoxide anion combined with the nitric oxide (NO), by the enzyme NO synthase, can generate peroxinitrite ($ONOO^-$), which has a great oxidant

power and cytotoxic activity (Rosen et al., 1995). NO is known to have a microbicidal activity against parasites, bacteria and viruses. NO constitutes an alternative method of killing invading pathogens (Tafalla et al., 2003). The ability of *R. decussatus* clams to produce NO in response to zymosan or bacterial lipopolysaccharide (LPS) was measured by Tafalla et al. (2003); their results showed an increase of NO production after exposure to zymosan, LPS and the pathogenic bacteria *Vibrio tapetis*. The production of NO was also independent from phagocytosis. Production of Nitric Oxide (NO) is increased in *C. virginica* haemocytes exposed to *P. marinus*; NO contributes in first term to elimination of *P. marinus* cells but the parasite overcome the NO damage in some term and the progression of the infection continues (Villamil et al., 2007).

I.2.2.6. Apoptosis

Apoptosis or Programmed Cell Death is a resource that the organisms can use to avoid proliferations of intracellular pathogens. With the death of the cells the pathogens are not liberated thus avoiding avoid their dispersion (Villalba et al., 2011). *P. marinus* inhibits the apoptosis of *C. virginica* haemocytes indicating the modulation of the host immune response (Sunila and LaBanca, 2003; Hughes et al., 2010). Instead of this, there were not significant changes in apoptosis rate in *R. philippinarum* haemocytes exposed to *P. olseni* cells (Hégaret et al., 2007; da Silva et al., 2008) which is consistent with the extracellular proliferation strategy of *P. olseni*.

I.3. PERKINSOSIS

I.3.1. General overview

Perkinsosis is the disease caused by the protozoan parasite *Perkinsus* spp. This disease has caused mass mortality of molluscs resulting in dramatic economic losses (Villalba et al., 2004). In 1950 John G. Mackin, H. Malcon Owen and Albert Collier discovered a parasite associated with high mortalities in the oyster *Crassostrea virginica* in the Gulf of Mexico (Mackin et al., 1950). In a first time this parasite was called *Dermocystidium marinum* and actually is called *Perkinsus marinus*. Since this first detection, several species of *Perkinsus* were found infecting a high variety of molluscs, bivalves and gastropods. This genus of parasites is present in North and South America, Europe, Africa, Asia and Australasia.

I.3.2. Life cycle

Parasites of the genus *Perkinsus* have three main cellular stages in the life cycle: trophozoite, hypnospore and zoospore.

The **trophozoite** stage occurs in the host. The cell in this stage is spherical and has a large vacuole and peripheral prominent nucleus with a nucleolus; thus, the cell has “signet ring” appearance (Fig. I.6); in some species, there is a polymorphic

inclusion in the vacuole called vacuoplast (Villalba et al., 2004). The size of the trophozoite varies among species. The proliferation of the parasite through host tissues takes place by vegetative proliferation (palintomy) (Fig.I.7); trophozoites are divided by successive bi-partitioning (cycles of karyokinesis followed by cytokinesis) to yield up to 32 daughter cells that stay together inside the mother wall; eventually, the wall brakes and the daughter cells are liberated; the resulting immature throphozoites gradually enlarge and form the vacuole inside becoming mature trophozoites (Perkins, 1996; Villalba et al., 2011).

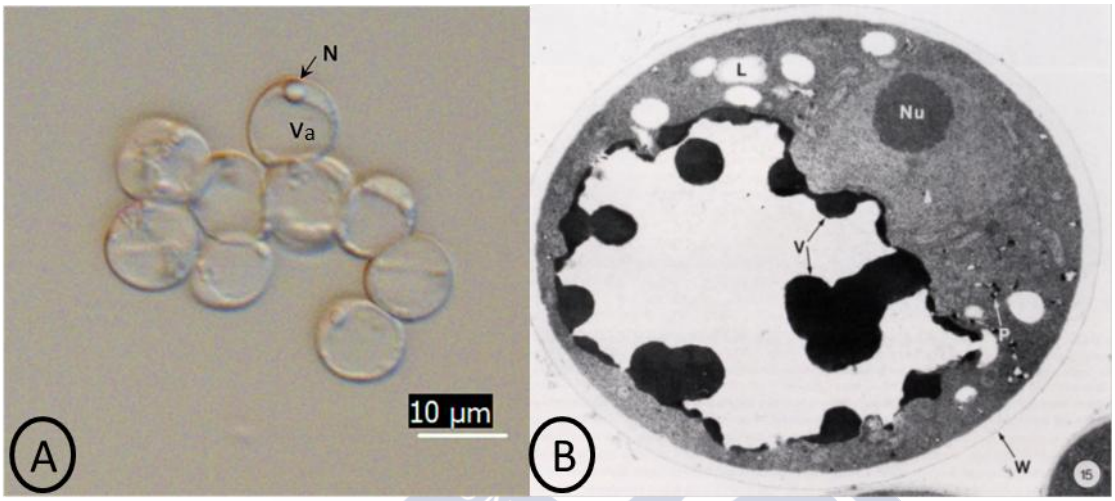

Fig. I.6. Images of trophozoites of *Perkinsus* spp. A. *Perkinsus chesapeaki in vitro*-cultured mature thophozoites. The micrograph shows the typical "signet ring" appearance with the prominent nucleus (N) and the large vacuole (Va). B. Transmission electron microscopy micrograph of a mature throphozoite from a *Perkinsus marinus* culture. L: lipid dropet; N: nucleus; Nu. nucleolus; P: presuntive precursor material of vacuoplast in cisternae of the endoplasmatic reticulum; V: vacuoplast material inside the eccentric vacuole; Va: Vacuole; W: cell wall; white arrowhead: cluster of intranuclear virus-like particles. The image B is taken from Perkins (1996).

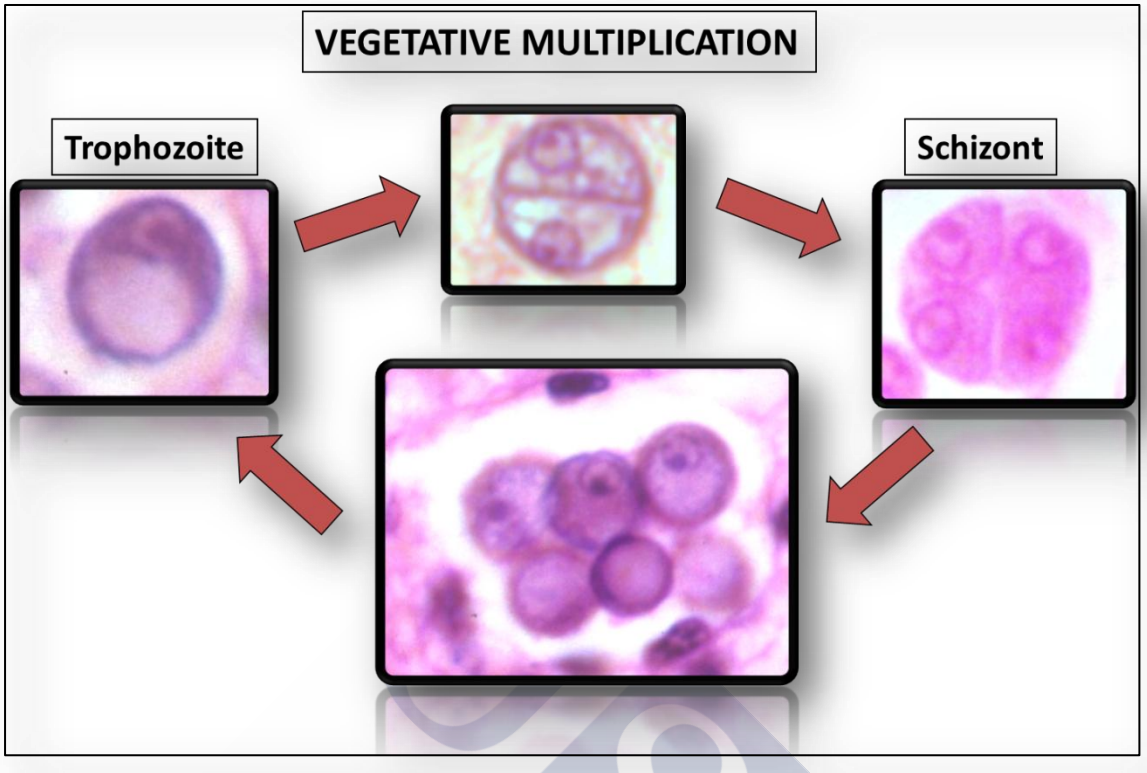

Fig.I.7. Micrographs showing progressive steps of vegetative multiplication of *Perkinsus olseni*. The trophozoite is divided in two and then four cells, which become united within the wall of the mother cell (schizont); eventually, that wall breaks and young trophozoites become free.

The parasite has a dormant/resistant stage that is produced when the host dyes or is dying. Host death involves changes of environmental conditions in the host tissue, such as low oxygen, acidic pH and increased nutrient levels, change; when that occurs, thophozoites enlarge keeping the spherical shape and develope a thick wall; this stage is called **hypnospore** (Casas and La Peyre, 2013). If the infected host tissues are incubated in fluid thioglycollate medium (FTM), trophozoites also transfrom into hypnospores (Ray, 1952). Hypnospores can live for long periods waiting for suitable conditions to address the next stage of the life cycle, zoosporulation (Chu and Greene, 1989; Casas et al., 2002a). When hypnospores reach the water column and the environmental conditions (temperature and salinity) are suitable, the zoosporulation occurs through successive karyokinesis and cytokinesis to form hundreds of cells within the original thick cell wall; thus the hypnospore becomes a zoosporangium and the daughter cells become zoospores that are released through a discharge tube, which is formed very early in the zoosporulation process, even before the first cell division takes place (Fig. I.8). Zoospores are ellipsoidal and biflagellated (Fig. I.9) (Perkins and Menzel, 1967; Casas et al., 2002a; Villalba et al., 2004).

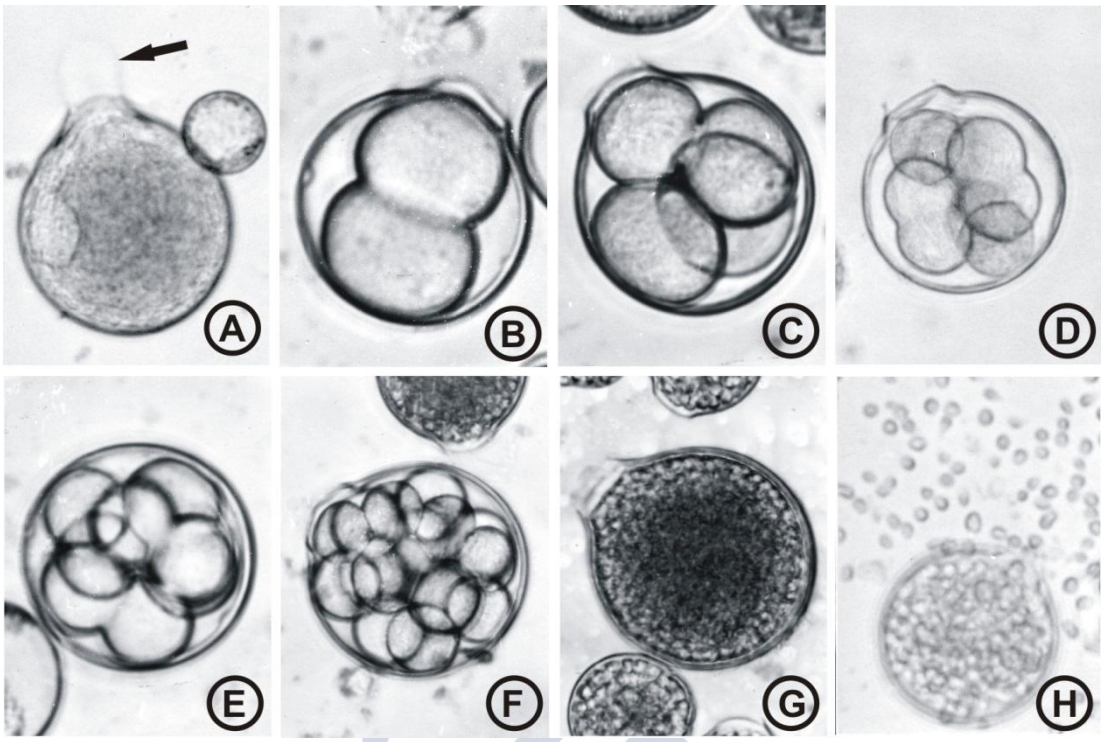

Fig. I.8. Progression of zoosporulation. A. Unicellular zoosporangia with the discharge tube (arrow) formed. B. Two-cells zoosporangium. C. Four-cells zoosporangium. D. Zoosporangium cell division between 4 to 8 cells. E. Eight-cells zoosporangium. F. Sixteen-cells zoosporangium. G. Hundreds of motile zoospores inside the zoosporangium. H. Release of the zoospores through the discharge tube. Images taken from Villalba et al. (2011).

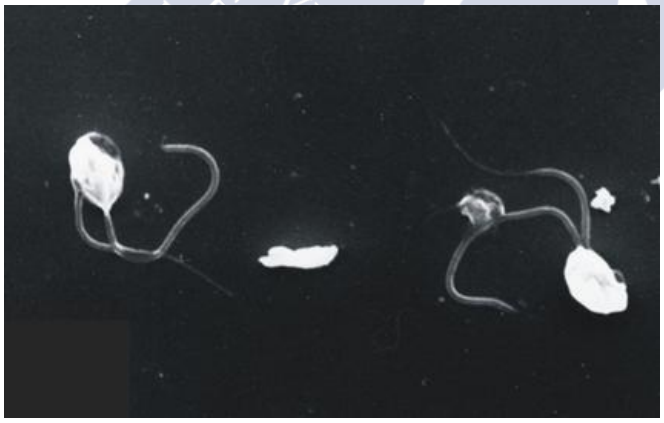

Fig. I.9. Transmission electronic microscopy micrograph of two *Perkinsus olseni* zoospores showing the ellipsoidal shape and the two flagella. Taken from Villalba et al., (2011).

Transmission of *Perkinsus* sp. is direct mollusc to mollusc without intermediate host (Ray, 1954; Chu, 1996). After the death of the host, the zoospores released to the water can infect new molluscs (Chu, 1996; Ford et al., 2002) but new infections are possible without the death of the host. Trophozoites are released to the water through the faeces (Bushek et al., 2002; Park et al., 2010) and probably through the gonoducts (Moss et al., 2008), which can infect new hosts. The maximum transmission rate occurs when the mortality of the molluscs reaches a maximum, but the transmission is also

produced when there is not mortality, so the death of the host is not necessary for the dispersion of the disease (Ragone Calvo et al., 2003). The proximity among individuals increase the possibility of transmission (Andrews, 1965). A scheme of the life cycle of the parasites of the genus *Perkinsus* is shown in Fig. I.10.

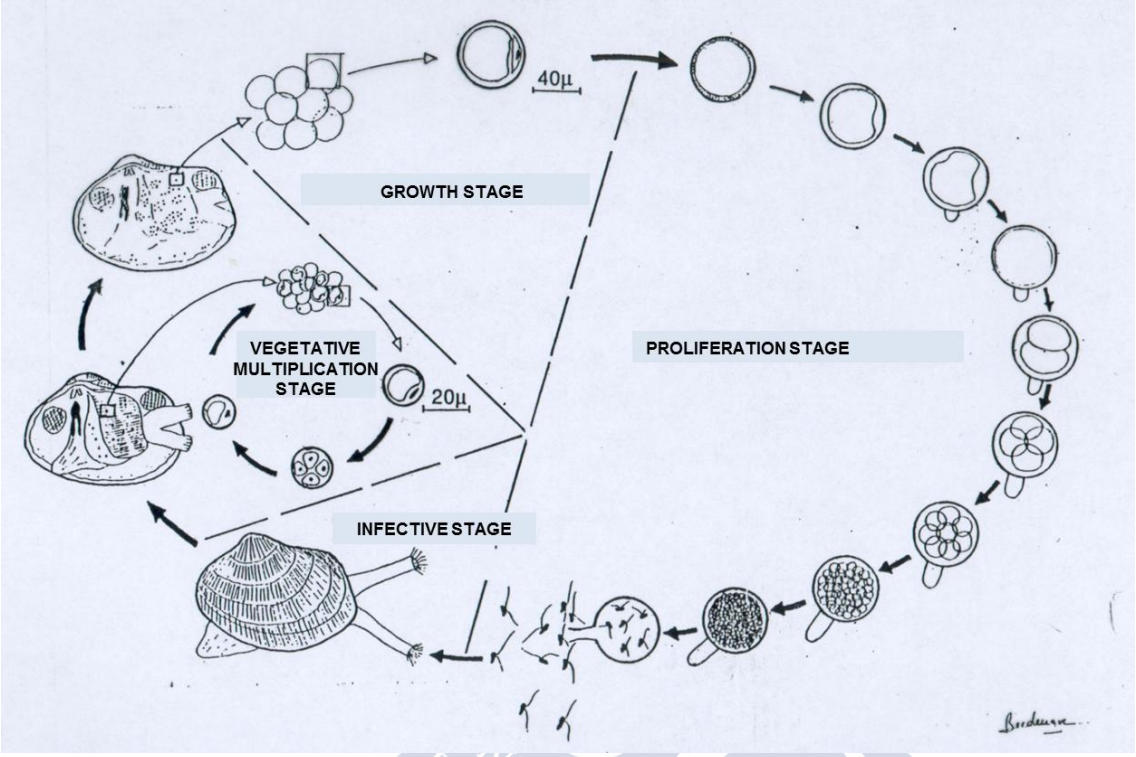

Fig. I.10. Life cycle of *Perkinsus olseni* parasitising clams. Image taken from Auzoux-Bordenave et al. (1995).

I.3.3. Taxonomy and phylogeny

The first parasite discovered of this group was described as a fungus, included in the genus *Dermocystidium* and called *Dermocystidium marinum* (Mackin et al., 1950). A new morphological study of the parasite concluded that it was a protozoan, and was included into the phylum Labyrinthomorpha as *Labyrinthomyxa marina* (Mackin and Ray, 1966). Later, according to the zoospore structure, the presence of the cortical alveoles, micropores and the conoid apical structure, the taxonomic position changed to the phylum Apicomplexa (Perkins, 1976). Then, based on this unique structure, Levine (1978) established the new genus *Perkinsus*, placing it within Apicomplexa. With the development of the molecular techniques, new taxonomic studies were done as molecular techniques were available; the analysis of the DNA sequence of the small subunit of the rRNA (SSU rRNA) showed that *Perkinsus* is closer to Dinoflagellates than to Apicomplexa (Goggin and Barker, 1993; Siddall et al., 1997). Studies based on the nuclear-encoded spliced leades (SL) RNA and mitochondrial genes, intron prevalence, and multi-protein phylogenies expanded support for the affiliation of the genus *Perkinsus* with an independent lineage (Perkinsozoa),

positioned between the phyla of Apicomplexa and Dinoflagellata (Zhang et al., 2011). The identification of plastid-associated major biosynthetic pathways in *Perkinsus* suggests the presence of a relic plastid. This plastic could suggest a common ancestor in Chromalveolata branch (nowadays Alveolata branch) as Fernandez-Robledo et al. (2011) hypothesised. Nowadays, genus *Perkinsus* is included into a supergroup defined as SAR (Fig. I.11), which name is derived from the acronym of the three groups united in this clade - Stramenopiles, Alveolata and Rhizaria -, and included into the Alveolata group, a group that includes Protalveolata, Dinoflagellata, Apicomplexa, Cilophora, Ellobiopsidae and Colponema (Saldarriaga et al., 2003; Cavalier-Smith and Chao, 2004; Adl et al., 2005; Adl et al., 2012).

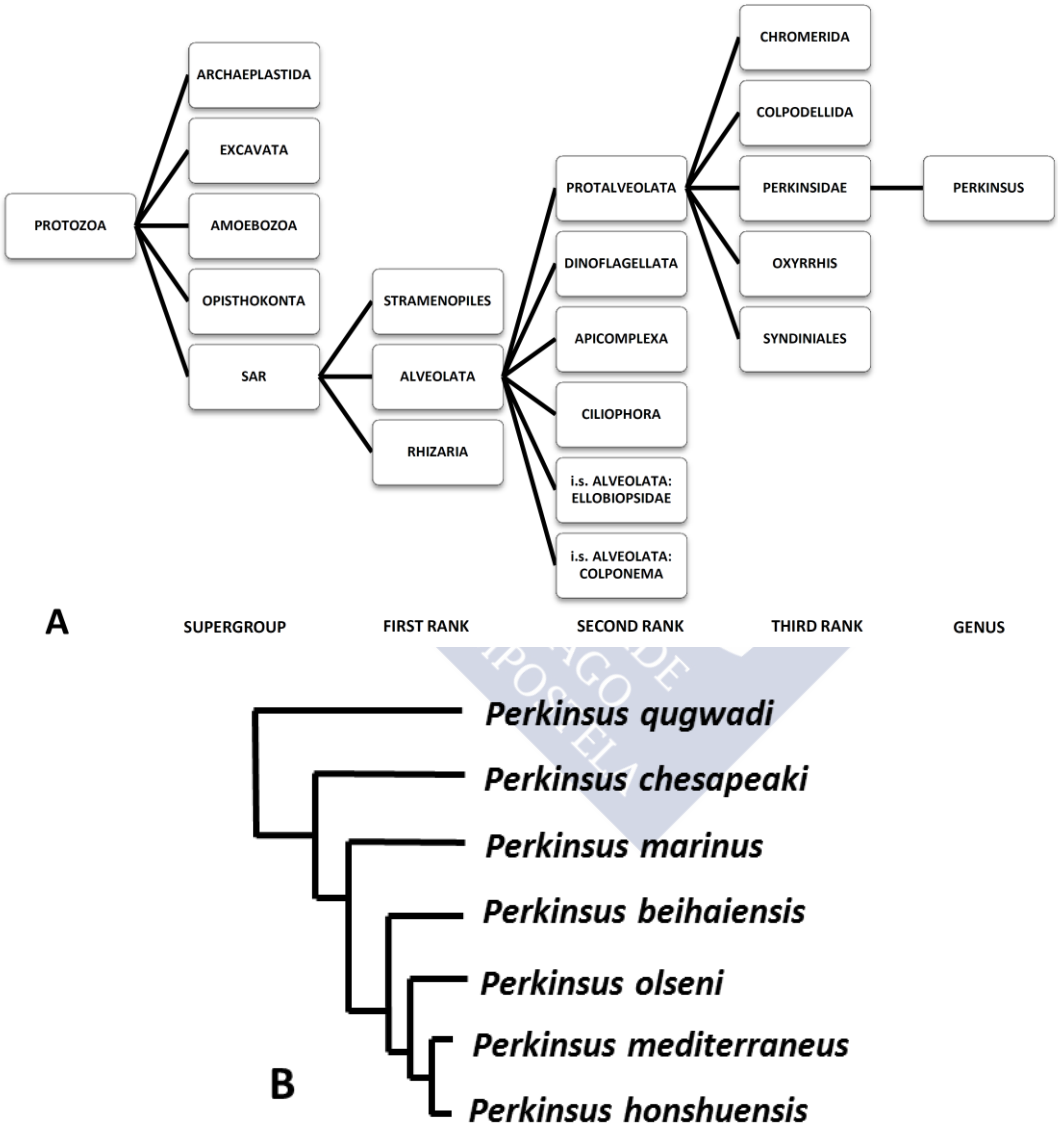

Fig. I.11. A. General view of the taxonomic position of the genus *Perkinsus*, according to the International Society of Protozoologists (Adl et al., 2012) where i.s. means "incertae sedis". **B.** Phylogenetic relationships of the *Perkinsus* species based on ITS region of the rRNA gene, according to da Silva et al. (2014).

Since the discovery of *Perkinsus marinus* in 1950, more species have been described and 7 species are currently accepted within this genus. Morphological characters of *Perkinsus* do not allow discriminating species, thus taxonomy of the genus is mainly based on gene sequences, particularly those of the different regions of the rRNA gene but also actin type I and II (Dungan and Reece, 2006; Moss et al., 2008). The second discovered species of this genus was *Perkinsus olseni*. It was described in the abalone *Haliotis rubra* in Australia by Lester and Davis (1981). Later, a new species called *Perkinsus atlanticus* was described in carpet-shell clams *Ruditapes decussatus* from Portugal (Azevedo, 1989). However, due to the homology of the rRNA gene sequence, *P. atlanticus* was considered synonymous of *P. olseni* (Murrell et al., 2002). Blackbourn et al. (1998) described *Perkinsus qugwadi* as the agent of high mortality of the scallop *Pactinopecten yessoensis* in Canada. Two species of the genus were described in the period 2000 - 2001, in Chesapeake Bay, *Perkinsus chesapeaki*, infecting the soft-shell clam *Mya arenaria* (McLaughlin et al., 2000), and *Perkinsus andrewsi* in the Baltic clam *Macoma balthica* (Coss et al., 2001) but, again, *P. andrewsi* was considered synonymous of *P. chesapeaki* (Burreson et al., 2005). Casas et al. (2004) described *Perkinsus mediterraneus* infecting the European flat oyster *Ostrea edulis* in Mediterranean waters. Recently, two more species have been described in Asia, *Perkinsus honshuensis* infecting the Manila clam *Ruditapes philippinarum* in Japan (Dungan and Reece, 2006) and *Perkinsus beihaiensis* infecting the oysters *Crassostrea hongkongensis* and *Crassostrea ariankensis* in China (Moss et al., 2008). The current geographic distribution of *Perkinsus* spp. is shown in Fig. I.12. The information on valid species of *Perkinsus* spp. and their known hosts is summarised in Table I.2.

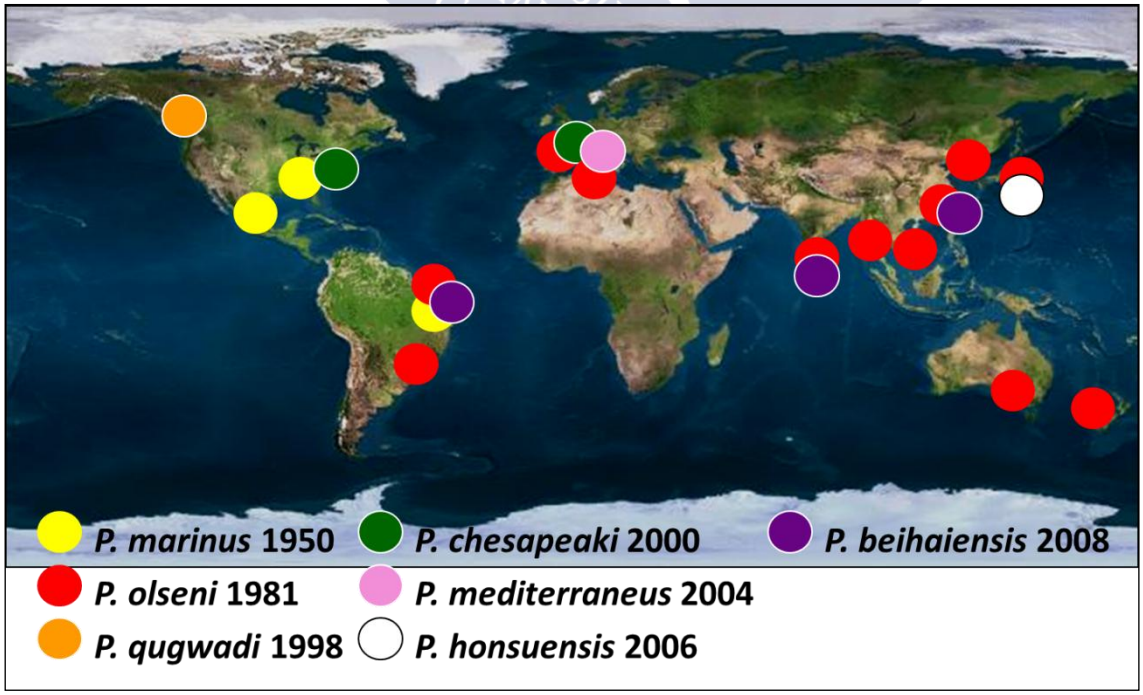

Fig. I.12. Geographic distribution area of the *Perkinsus* spp.

Table I.2. Valid species of the genus *Perkinsus*, geographic distribution and their host

Species	Type host	Other hosts	Affected countries	References
Perkinsus marinus	*Crassostrea virginica*	Oysters: *Crassostrea gigas*, *Crassostrea ariakensis*, *Crassostrea rhizophorae, Crassostrea corteziensis, Crassostrea gasar, Saccostrea palmula* Clams: *Mya arenaria*, *Macoma balthica*, *Macoma mitchelli*, *Mercenaria mercenaria*	USA, Mexico, Brazil	Mackin et al., 1950; Burreson et al., 1994; Andrews, 1996; Calvo et al., 1999; Calvo et al., 2001; Coss et al., 2001; Cáceres-Martínez et al., 2008; Pecher et al., 2008; Cáceres-Martínez et al., 2012; da Silva et al., 2013; da Silva et al., 2014.
Perkinsus olseni (= *atlanticus*)	*Haliotis rubra*	Clams: *Ruditapes decussatus, Ruditapes philippinarum*, *Austrovenus stutchburyi*, *Venerupis corrugata,Venerupis aurea, Tapes rhomboides*, Anadara trapecia, *Pitar rostrata*, *Protothaca jedoensis, Paphia rhomboides; Paphia aurea, Paphia undulata, Cerastoderma glaucum* Oysters: *Crassostrea ariakensis*, *Crassostrea hongkongensis*, *Crassostrea rhizophorae*, *Crassostrea gasar, Pinctada máxima, Pinctada fucata* Abalones: *Haliotis laevigata*, *Haliotis scalaris*, *Haliotis cyclobates*	Australia, New Zealand, South Korea, Japan, China, Thailand, India, Portugal, Spain, Italy, Tunisia, Uruguay and Brazil	Lester and Davis, 1981; Azevedo, 1989; Norton et al., 1993; Goggin and Lester, 1995; Hamaguchi et al., 1998; Fernández-Robledo et al., 2000; De la Herrán et al., 2000; Leethochavalit et al., 2004; Cremonte et al., 2005; Park et al., 2005; Park et al., 2006; Zhang et al., 2005; Abollo et al., 2006; Moss et al., 2007; Sanil et al., 2010; El Bour et al., 2012; Ramilo et al., 2012; da Silva et al., 2014; Pretto et al., 2014; Ramilo et al., 2015.
Perkinsus qugwadi	*Patinopecten yessoensis*		Canada	Bower et al., 1998; Itoh et al., 2013.
Perkinsus chesapeaki (= *andrewsi*)	*Mya arenaria*	Clams*: Macoma baltica*, *Macoma mitchelli*, *Mercenaria mercenaria*, *Tagelus plebeius*, *Cyrtopleura costata*, *Rangia cuneata*, *Mulinia lateralis; Ruditapes decussatus, Ruditapes philippinarum* Cockles: *Cerastoderma edule* Oysters: *Crassostrea virginica*	USA, Spain, France	Kotob et al., 1999; Mc Lauglin et al., 2000; Coss et al., 2001; Dungan et al., 2002; Burreson et al., 2005; Reece et al., 2008; Arzul et al., 2012; Ramilo et al., 2012; Carrasco et al., 2014.
Perkinsus mediterraneus	*Ostrea edulis*	Clams: *Chamaelea gallina, Venus verrucosa, Arca noae.* Pectinids*: Chlamys varia*	Spain	Casas et al., 2004; Moss et al., 2008; Ramilo et al., 2015.
Perkinsus honshuensis	*Ruditapes philippinarum*		Japan	Dungan and Reece, 2006.
Perkinsus beihaiensis	*Crassostrea hongkongensis*	Oysters: *Crassostrea ariakensis, Crassostrea madrasensis* Clams*: Anomalocardia brasiliana*	China, India, Brazil	Moss et al., 2008; Sanil et al., 2012; Ferreira et al., in press

The World Organisation for Animal Health (OIE) has included two species of this genus, *P. marinus* and *P. olseni*, in the list of notifiable diseases (http://www.oie.int/ en/ international-standard-setting/ aquatic-code/access-online/) because of their known pathogenicity.

Taxonomic review within genus *Perkinsus* has to include the occurrence of races or strains within species. It was first elucidated by Reece et al. (1997) after studying four polymorphic loci of *Perkinsus marinus* from nine geographically distant sites. All loci were variable within and among *P. marinus* cultures, suggesting that cultures could have different genetic characteristics that explained the physiological differences between isolates of *P. marinus*, such as virulence (Bushek and Allen, 1996) or production of extracellular proteins (La Peyre et al., 1996) that could represent differences in virulence (La Peyre and Faisal, 1995; La Peyre et al., 1995b, 1996). *P. marinus* northern and southern populations were found to be genetically distant (Reece et al., 1997), suggesting the possibility of cold-tolerant strains of *P. marinus*. Robledo et al. (1999) examined the sequence variability of the nontranscriber spacer (NTS) and the internal-transcriber spacer (ITS1 and ITS2) domains of the rRNA locus of *P. marinus* from three populations of USA coast. The results shown great differences in genotype frequencies among populations, supporting the existence of *P. marinus* strains. Two years later, Recce et al. (2001) enlarged their first research of 1997 and studied more than 150 cultures of *P. marinus* from different locations with eight polymorphic loci. This research provided evidences of different allelic and genotypic frequencies among three regions of the USA; the Northeast Atlantic (Maine to Maryland), the Southeast Atlantic (Virginia to Florida's east coast), and the Gulf of Mexico (Florida's west coast to Texas). Thompson et al. (2014a) analysed the genetic structure of *P. marinus* from 15 locations of USA, from Gulf of Mexico to Massachusetts, with 7 microsatellite loci. Allele frequencies did not show isolation by distance but four distinct clusters of multilocus genotypes were identified. Some of the lineages were limited to a particular region or disjunct locations and two lineages were associated with recent range expansion. This study amplified the results obtained in 2011 for the same group (Thompson et al., 2011) in which 7 microsatellite loci differentiate 126 *P. marinus* strains unique among 129 genotypes from four locations, revealing remarkable genetic diversity and both asexual and sexual reproductive processes. These strains could have differences in virulence or response to host defences; *P. marinus* isolates showed different patterns of response after testing of several anti-malaria drugs, supporting the concept of strain/species variability (Alemán-Resto and Fernández-Robledo, 2014). In summary, different *Perkinsus* races or strains occur, which could have differences in virulence, resistance to host defense attack or endure extreme conditions, thus allowing the parasite colonising new locations.

I.3.4. Epidemiology

Several factors influence intensity and prevalence of the infection.

- *Influence of environmental factors*

The influence of temperature and salinity in the intensity and prevalence of the infection by *Perkinsus* is well known. Field studies have shown that the infection of oysters *C. virginica* with *P. marinus* is favoured by high salinity (Ray, 1954; Mackin and Boswell, 1956; Soniat, 1985; Soniat, 1996), whereas the infection level is low or even inexistent in low salinity areas; the annual temperature cycle induces a seasonal pattern of the disease (Andrews and Ray, 1988; Burreson and Ragone-Calvo, 1996; Soniat et al., 2005; La Peyre et al., 2008). In *P. olseni* case, the study of the infection in a natural clam *R. decussatus* seabed in Galicia showed an annual pattern influenced by temperature (López et al., 1998; Villalba et al., 2005); in spring, when the temperature raises above 15 °C the prevalence and intensity of the infection also raises; in summer the intensity continues high and it is at the end of summer or beginning of autumn (temperature 19 - 21 °C) when mortality reaches the highest value; as temperature decreases, the intensity and prevalence of the infection goes down and it is in winter, with temperature about 9 - 10 °C, when the lowest values of prevalence and infection intensity occur. Nevertheless, seasonal patterns were not clear in some studies. In Arcachon Bay (France), with a prevalence of perkinsosis of 93%, no seasonal cycle was evident. The combination of episodic infection events and slow disinfection kinetics could explain the lack of concordance among seasons (Dang et al., 2010).

- *Stressing factors*

Degradation of environmental conditions and habitat destruction favour infection (Chu and Hale, 1994). Park and Choi (2001) found higher prevalence of *P. olseni* infections in *R. philippinarum* grown in silty-mud beds than those grown in sandy beds. *P. olseni* prevalence in Cheju Island (South Korea) was limited to the clam populations distributed in inner harbors where water and sediment pollution level is high and sea current low (Park and Choi 2001). Density of clams is another factor which could affect to prevalence; higher density of clams can enhance the possibility to be infected (Santmartí et al., 1995; Choi and Park, 2010; Casas and Villalba, 2012), this together with events of hypoxia and contamination can provoke mass mortalities (Villalba et al., 2011). The nutritional stage and the weakness of the host after the spawn could also affect infection progression (Hofmann et al., 1995; Powell et al., 1996).

- *Age influence*

The prevalence of *P. marinus* is higher in adult oysters *C. virginica* than in juveniles because of the the higher filtration rate and longer exposure to the parasite

of adults (Burreson and Ragone-Calvo, 1996; Powell et al., 1996; Soniat, 1996). A similar association between age and infection of *R. decussatus* with *P. olseni* has been described. In a natural clam *R. decussatus* bed of Galicia, all the clams above 3 years old were infected while none of the clams with less than a year old appeared infected (Villalba et al., 2005). Similar results were reported in Korea, no infection was found in clams *R. philippinarum* < 15 mm length (< 1 year old) and nearly 100% infection was detected in clams over 20 mm (Choi and Park, 1997). Although in natural conditions juvenile clams appear to be non-infected, in experimental conditions juvenile clams are more susceptible to *P. olseni* than adult clams: juvenile Manila clams (3-10 mm length) showed a dose dependent mortality after challenge with *P. olseni* prezosporangia (Shimokawa et al., 2010). A comparison between juvenile and adult clams to exposure of *P. olseni* prezosporangia at different temperatures showed higher mortality in juvenile clams at every temperature value, authors concluded that the pathogenicity of the infection is influenced by environmental conditions and host age (Waki and Yoshinaga, 2013).

- *Predictive models*

Predictive mathematic models have been developed for the infection of the oyster *C. virginica* with *P. marinus*, taking into account the main factors that influence disease progression, namely water temperature and salinity (Powell et al., 1996; Soniat and Kortright, 1998; Ragone-Calvo et al., 2000).

I.3.5. Diagnostic methods

There are three main groups of diagnostic methods for perkinsosis: (1) observation of the parasite in the host tissues, (2) tissue incubation in fluid thioglycollate medium and (3) molecular techniques.

- *Observation of the parasite in the host tissues*

This group includes standard microscopic techniques as histological sections, haemolymph samples, tissue smears… Due to the difficulty to discriminate species by morphological characters, this group of techniques does not allow the identification of the species (Villalba et al., 2011).

- *Tissue incubation in tioglicolate medium*

This technique was developed in the 1950´s when researchers were trying to establish a culture of the parasite, then thought to be a fungus. This technique was developed by Ray (1952) who observed that, after incubation in fluid thioglycollate medium (FTM), *Perkinsus* cells enlarged and develop a wide wall (hypnospore stage). Ray (1966) himself modified the protocol and it has become a widely used procedure for diagnosis of perkinsosis. Hypnospores are deeply stained with Lugol's solution, so

their visualisation as large, dark spheres is very easy with light microscope. The protocol involves incubating tissue pieces of the mollusc in FTM for 7 days in the dark at room temperature. Then, tissues are set on a slide, chopped and stained with Lugol's solution. This is a quantitative diagnostic method because the parasite cells do not divide in the culture medium, they only increase in size. Therefore, the number of dark, large spheres observed in the tissue pieces after incubation exactly corresponds to the number of trophozoites occurring in the tissues before incubation. A scale ranking the infection intensity in 7 classes was established by Mackin (Ray, 1954), with numerical value of 0, 0.5, 1, 2, 3, 4 or 5 for null, very light, light, light to moderate, moderate, moderate to heavy, and heavy infections, respectively. The assignation of the infection intensity to one of these classes was based on counting the number of hypnospores in a number of microscope fields. This diagnostic method is cheap and simple, requiring reduced equipment and training, and more sensitive than standard histology because higher tissue volume is scanned; however it does not allow identifying the species of *Perkinsus*. The infection in the host is not homogeneously distributed throughout all the organs, because of this the use of the whole body was proposed for a more accurate estimation of the infection, usually expressed as the number of hypnospores per gram of host tissue (Choi et al., 1989; Fisher and Oliver, 1996; Almeida et al., 1999). The FTM was also adapted by incubating host haemolymph as a non-lethal diagnostic way (Gauthier and Fisher, 1990).

- *Molecular methods*

Several molecular diagnostics assays have been developed through the years. The most used ones are based on the DNA sequence of the rRNA gene. Some PCR primers have been designed specific for only one species as *P. marinus* (Fernández-Robledo et al., 1998), *P. chesapeaki* (Coss et al., 2001), *P. olseni* (Hamaguchi et al., 1998; Fernández-Robledo et al. 2000), *P. beihaiensis* (Moss et al., 2008) and recently for *P. qugwadi* (Itoh et al., 2013). Other primers were designed with specifity for the genus *Perkinsus*, based either on the internal transcribed space (ITS) sequence (Casas et al., 2002a) or the intergenic spacer (IGS) sequence (Fernández-Robledo et al., 2002). Restriction fragment length polymorphism (RFLP) assays to be applied on amplicons from genus-specific PCRs were developed to discriminate *Perkinsus* spp. (Kotob et al., 1999; Abollo et al., 2006). *In situ* hybridisation methods with DNA probes specific for the genus *Perkinsus* (Elston et al., 2003) or specific for one species, such as *P. olseni* (Navas et al., 2001) and *P. beihaiensis* (Moss et al., 2008), have also been developed. More recently, a nested PCR was developed based on ITS-1 and ITS-2 regions of the RNA using the primers described by Kotob et al. (1999) in the first PCR and specific primers in the second PCR (Balseiro et al., 2010). Real-time PCR was also developed to detect and quantify *P. marinus* (de Faveri et al., 2009), *P. olseni* and *P. honsuensis* (Umeda and Yoshinaga, 2012) and dual infections of *Haplosporidium* spp. and *Perkinsus* spp. (Xie et al., 2013). Also, a diagnostic method using the technique loop-

mediated isothermal amplification (LAMP) was designed for *Perkinsus* spp. and utilised to detect *P. olseni* and *P. marinus* infections (Feng et al., 2013).

I.3.6. In vitro *culture of* Perkinsus *spp.*

The availability of large quantities of parasites makes easier the investigation of the disease. Since the development of a culture media in 1993 the number of publications about *Perkinsus* spp. rises exponentially (Fernández-Robledo et al., 2014). Some aspects of the parasite life cycle could not be elucidated without the culture of the species. One of the first culture media for *Perkinsus* spp. was developed by La Peyre et al. (1993) using a composition similar to that of oyster plasma; that culture medium, called JL-ODRP-1, was first used for the proliferation of *P. marinus* (La Peyre et al., 1993) and later for *P. chesapeaki* (McLaughlin and Faisal, 1998). An improved version of the culture medium, JL-ODRP-2, was used to culture *P. olseni* (Casas et al., 2002b) and a protein-free culture medium based on JL-ODRP-2 called JL-ODRP-2F was used to establish *P. mediterraneus* cultures (Casas et al., 2008; Casas et al., 2011). Another culture media, the commercial DME: Ham´s F-12 with nutrient supplements, was used for the proliferation of *Perkinsus marinus* (Gauthier and Vasta, 1993, 1995; Gauthier et al., 1995), *P. chesapeaki* (Coss et al., 2001; Burreson et al., 2005), *P. olseni* (Casas et al., 2002b; Robledo et al., 2002; Dungan et al., 2007), *P. honsuensis* (Dungan and Reece, 2006) and *P. beihaiensis* (Moss et al., 2008). *P. marinus* was also cultures in Leibowitz´s L-15 supplemented media (Kleinschuster and Swink, 1993). In order to study the extracellular products of *P. marinus* La Peyre and Faisal (1996, 1997a) designed media without protein content. The only species without an established culture is *P. qugwadi*. To start a culture, different organs of the host were used, heart, gills, visceral ganglia, haemolymph or even hypnospores isolated from the FTM.

Two procedures to assure that all the *Perkinsus* parasites proliferating *in vitro* are genetically identical (clonal cultures) have beed designed. The first one is based on serial dilution of the cells occurring in an *in vitro* culture and then plating a small volume in 96-well culture plates. After an examination under light inverted microscope, wells with only one cell are chosen and growth factors added; the proliferation of this cell originates a clonal culture (Gauthier and Vasta, 1995). The second method is based on micromanipulation; it involves isolating a single cell by micromanipulation, which is transferred into an insert with a permeable bottom, set on the well of the original culture. By this way, the extracellular products released by the cells in the original culture enhance the proliferation of the isolated cell, thus initiating a clonal culture (Casas and La Peyre, 2009). It is possible to measure the proliferation of the protozoan parasite in culture conditions using a bioluminescence assay for ATP content. This method is useful to assess the proliferation using different compounds in order to develop new culture media or to assess cell proliferation under potential anti-proliderative compounds (Shridar et al., 2013).

I.3.7. Interaction Perkinsus – *clam*

As mentioned above, *Perkinsus olseni* causes mortality in clams (Shimokawa et al., 2010; Waki et al., 2012; Pretto et al., 2014). High infection intensities (millions of parasites per gram of wet weight) can result in the death of the host, and causing direct economic and ecological consequences (Soudant et al., 2013). Additionally, sub-lethal effects of *Perkinsus* spp. infections may have a negative economic impact. Most studies on the effects of perkinsosis on host physiology are based on the infection of *C. virginica* with *P. marinus*. Adults of *C. virginica* heavily infected with *P. marinus* had a negative energetic balance because the energy consumed by the parasites was higher than the necessities of the oysters; this impact was lower in young oysters because of their lower respiration rate (Choi et al., 1989). A similar effect was observed in clams *R. decussatus* heavily infected with *P. olseni* (Casas, 2002). The effects on reproduction of *R. philippinarum* infected by *P. olseni* were evaluated and spawning frequency and oocyte production were negatively affected (Park et al., 2006). In *R. decussatus*, the inflammatory reaction caused by *P. olseni* infection affected the volume fraction of the gonadal components by increasing the volume fraction occupied by haemocytes and reducing the volume fraction of gametes, intrafollicular empty space, follicular walls and normal connective tissue, although but no significant effect on host gonadal index, fecundity or spawning efficiency was detected (Casas and Villalba, 2012). The energetic cost of heavy *Perkinsus* spp. infections may cause slower growth and loss of condition of the host (Andrews, 1961; Craig et al., 1989), which in the case of the clams is significant only at high infection intensity (Leite et al., 2004; Flye SainteMarie et al., 2009). All these combined effects reduce defense capacity to fight other pathogens. *P. olseni* infection favours other infections caused by viruses and bacteria in *R. philippinarum* (Montes et al., 2001; Dang et al., 2013, Soudant et al., 2013).

I.3.7.1. Clam response against Perkinsus olseni

Perkinsus olseni provokes an inflammatory response, based on infiltration and accumulation of haemocytes in the infected area to encapsulate the parasite cells (Chagot et al., 1987; Montes et al., 1995a; Casas and Villalba, 2012). Progression of the disease to heavy infections cause large granulocytomas distributed through the clam body causing the lost of organ structures. In the case of gonad, ganulocytomas invade the area occupied by follicles reducing the reproductive area (Casas and Villaba, 2012).

According to Montes et al. (1995b, 1996), connective tissues of infected *Ruditapes decussatus* clams can drive a specific inflammatory response. Granulocytes of clams are recruited into infected tissues where they differentiate into secretory granulocytes and synthesise a glycosylated polypeptide of 225kDa (Fig. I.13). This polypeptide is the main component of the capsule in which *P. olseni* trophozoites are encapsulated, but it is not expressed in *P. olseni* free clams or in clams exposed to other microorganisms (Montes et al., 1995b, 1996).

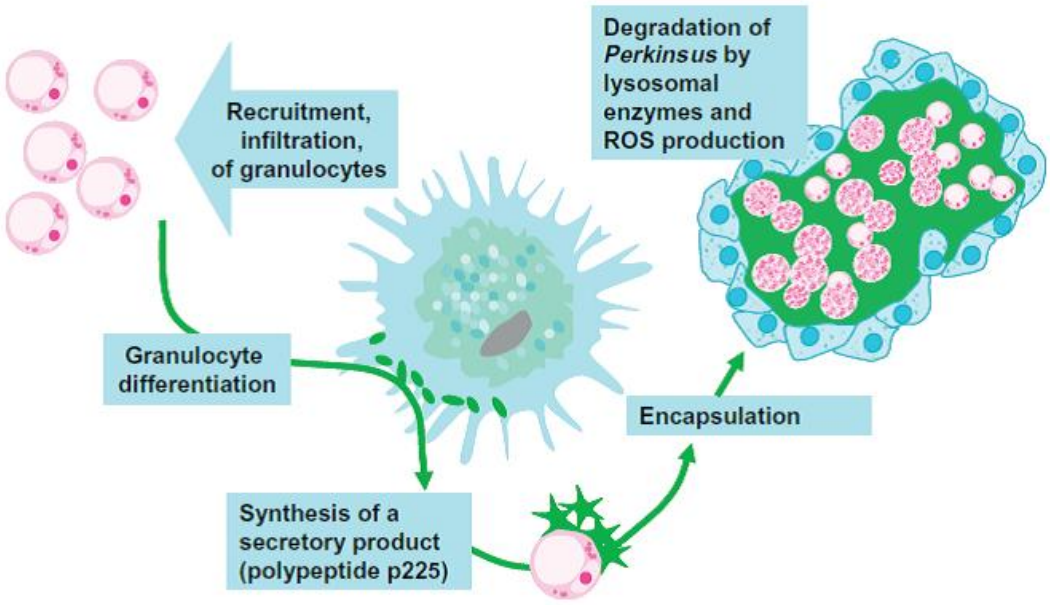

Fig. I.13. Schematic representation of *P. olseni* encapsulation by clam *R. decussatus* and *R. philippinarum* haemocytes. Image taken from Soudant et al. (2013).

Although it is unclear how efficiently can be the cells of *Perkinsus* spp. degraded or eliminated, the encapsulation process could block trophozoite dissemination (Rodriguez and Navas, 1995; Montes et al., 1995a). López et al. (1997c) confirmed that *R. decussatus* haemocytes can phagocytose *P. olseni* trophozoites in *in vitro* assays, whereas *P. olseni* zoospores were not phagocytosed. The involvement of lectins in the recognition and opsonisation of *P. olseni* cells within the immune response of clams has been described in section 2.2. The process of engulfment of *P. olseni* trophozoites by clam haemocytes is shown in Fig. I.14.

Some studies have been performed to improve the knowledge of the genes involved in the immune response against *P. olseni* in *R. decussatus* clams. Several genes related with immunity have been described by supression sustractive hybridisation (SSH) (Prado-Alvarez et al., 2009) and microarray at a transcriptomic level (Leite et al., 2013).

I.3.7.2. Parasite virulence factors

Perkinsus spp. serine proteases responsible for virulence and anti-oxidant activities allowing the parasite to elude host defense have been described and characterised.

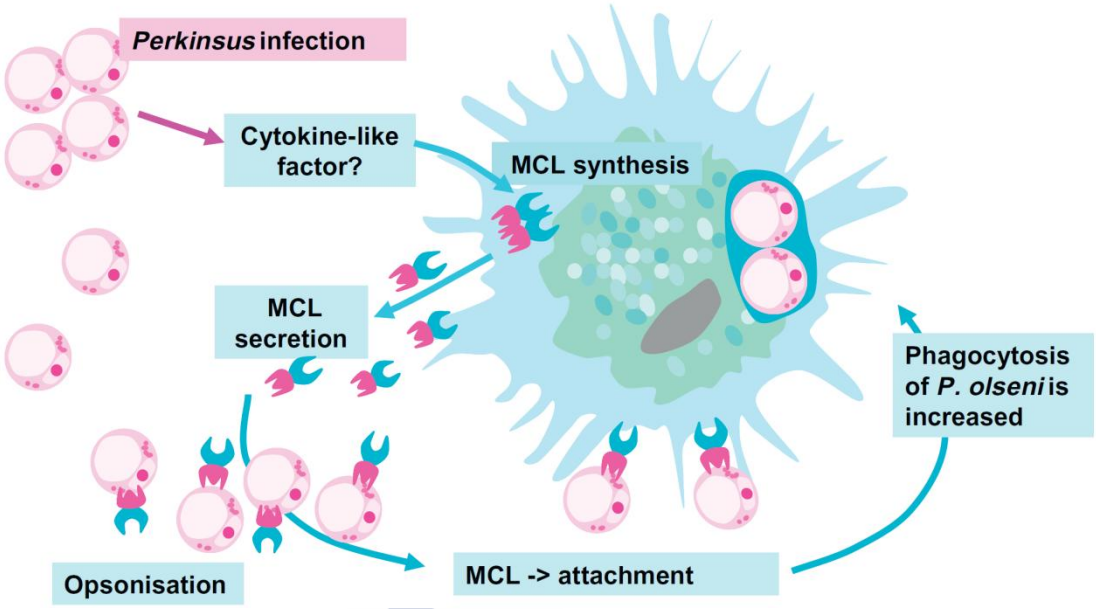

Fig. I.14. Hypothetical process of how Manila clam *Ruditapes philippinarum* lectins (MCL) are involved in recognition and opsonisation of *Perkinsus olseni* trophozoites for subsequent phagocytosis and elimination by clam haemocytes. Image taken from Soudant et al. (2013).

- *Lytic activity*

Extracellular products (ECPs) from *in vitro* cultured *P. marinus* were first studied by La Peyre et al., (1995a). *In vitro* experiments have shown that ECPs not only reduce several host haemocyte and humoral immune functions, including reactive oxygen species (ROS) and lysozyme production, cell mobility and haemagglutination (Garreis et al., 1996; Soudant et al., 2013), but also the bactericidal activity of haemocyte against *Vibrio* spp. bacteria (Tall et al., 1999). ECPs mainly consist of serine proteases, which have been proposed as possible virulence factors responsible for degradation of tissues of the infected oysters (La Peyre, 1996). In *P. marinus* the most abundant serine protease is a quimiotrypsin which was named as perkinsin (Faisal et al., 1999). The addition of eastern oyster homogenate to the culture medium specifically enhanced expression of serine proteases in *P. marinus* cultures (MacIntyre et al., 2003). Supplementation of *P. marinus* cultures with eastern oyster plasma or tissue homogenate also enhanced their infectivity (Earnhart et al., 2004). Serine protease activities such as trypsin and α-chymiotrypsin were found in ECPs of *P. marinus* but they were not in those of *P. olseni*, although other lytic activities as esterase, glucosidase and phosphatese were detected in ECPs of both species (Casas et al., 2002c). Several proteolitic bands with high differences among isolates from the same species were reported in ECPs of *P. mediterraneus* (Casas et al., 2008). Differences in proteolytic band profiles of culture supernadats between isolates of *P. marinus* had also been reported (La Peyre and Faisal, 1997b). A study that compared enzyme profiles in the supernatants of three *Perkinsus* spp. cultures (*P. chesapeaki*, *P. marinus*

and *P. olseni*) found hydrolytic activity in all the cultures but their relative concentration seemed to be unique to each species; *P. chesapeaki* supernatants showed proteolytic activity (Casas et al., 2009). No report on proteolytic activity in ECPs of *P. olseni* has been found in the revised literature.

- *Antioxidant capacity of* Perkinsus

Studies in confrontation of *C. virginica* haemocytes with *P. marinus* trophozoites showed an inhibition of ROS production by *C. virginica* haemocytes, otherwise, *P. marinus* still live with high concentrations of superoxide anion (O_2^-) and hydrogen peroxide (Schott et al., 2003). These observations suggest that *P. marinus* can inhibit ROS products or scavenge its products. Indeed, three anti-oxidant enzymatic activities involved in counteracting the oxygen dependent anti-microbial system have recently been identified and characterised in *P. marinus*: acid phosphatases (inhibition of O_2^- production), superoxide dismutases (neutralisation of O_2^-), and ascorbate peroxidases (H_2O_2 removal) (Volety and Chu, 1997; Wright et al., 2002, Schott et al., 2003; Schott and Vasta, 2003; Soudant et al., 2013) (Fig. I.15).

P. marinus was found resistant to the nitric oxide (NO) produced by oyster haemocytes. The parasite resisted high concentration of NO, thus evading its damaging effects (Villamil et al., 2007).

- *Parasite virulence genes*

With the availability of *Perkinsus* spp. cultures, several authors begun the search for genes related with virulence. One of the difficulties lies on the fact that the parasite quickly losses its virulence and pathogenicity when it is cultured under standard laboratory conditions (Volety and Chu, 1994; Bushek and Allen, 1996; Ford et al., 2002; Pales Espinosa et al., 2014). Wild-type *P. marinus* is significantly more virulent compared with cultured parasite cells (Ford et al., 2002). Despite loss of virulence in culture media, several genes related with virulence have been identified in the last years. Genes of two superoxide dismutases (PmSOD1 and PmSOD2) were reported in *P. marinus* (Schott and Vasta, 2003; Fernández-Robledo et al., 2008). The finding of these genes suggested the resistance of *P. marinus* to exogenous oxidative damage in host phagocytes.

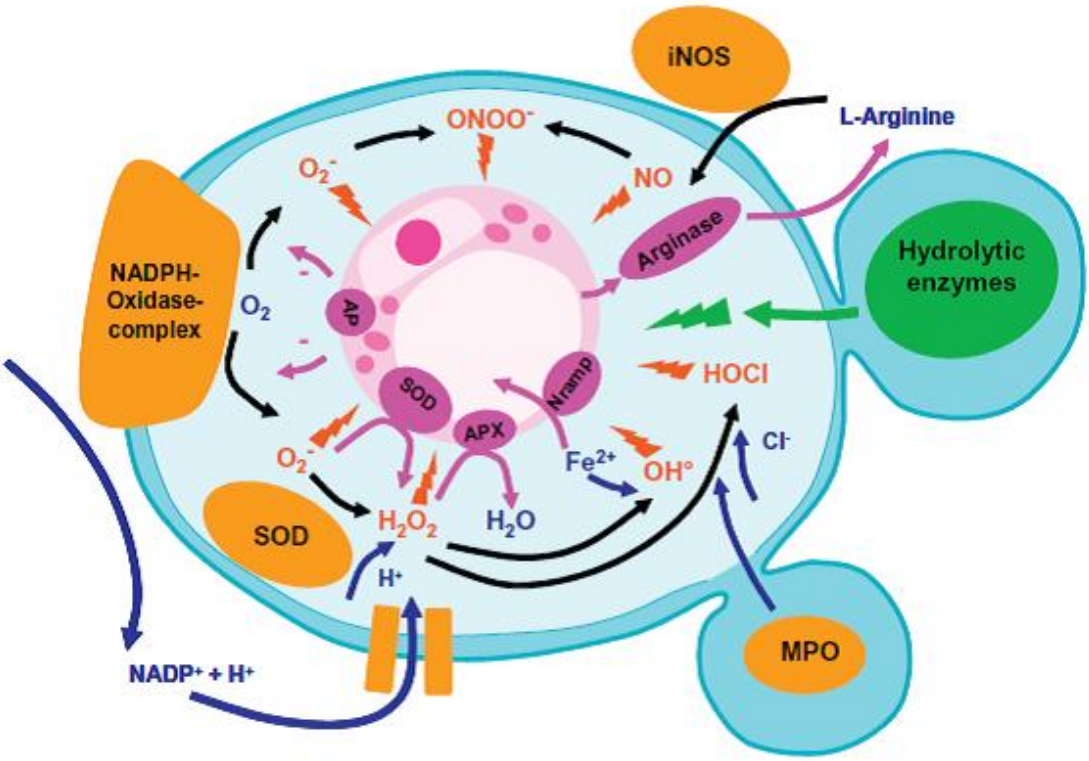

Fig. I.15. Antioxidant capacities of *Perkinsus marinus* upon phagocytosis by eastern oyster *Crassostrea virginica* haemocytes (phagosome is delimited by a blue line and phagocytoszed *Perkinsus* cell is pink). Toxic and non-toxic molecules are noted in red and blue, respectively. Pro-oxidant activities of haemocytes are represented in orange, and the anti-oxidant activities and mechanisms of *P. marinus* are represented in purple. Hydrolytic enzymes are represented in green. Abreviation: NO, Nitric oxide; $ONOO^-$, peroxynitrite; O_2^-, superoxideanion; HOCl, Hypochloride; iNOS, inducible nitric oxide synthase; SOD, superoxide dismutase; MPO, myeloperoxidase; AP, acid phosphatase; APX, ascorbate dependent peroxidase; Nramp, Natural Resistance-Associated Macrophage Protein. Image taken from Soudant et al. (2013).

Using universal primers, Brown and Reece (2003) isolated and characterised serine protease genes from *P. marinus,* which are involved in parasite evasion of host defense mechanisms (Chaudhuri et al., 1989). Leite et al. (2008) found high expression of hypoxia-inducible factors (HIF), mainly promoted by HIF proxyl hydrolases (HPHs), when cultures of *P. olseni* were supplemented with haemolymph from susceptible species to *P. olseni* infection; however, no significant expression of HPHs was found in cultures supplemented with haemolymph of resistant species to *P. olseni* infection, thus showing a high relationship between HPH gene expression and parasite virulence. A series of genes over-expressed in *P. olseni* confronted with *R. decussatus* haemolymph were identified by Ascenso et al. (2007). A comparison of these genes among *P. olseni* exposed to haemolymph from species with different susceptibility to *P. olseni* infection were measured by macroarray hybridisation (Ascenso et al., 2009).

A study of the effect of pallial mucus on *P. marinus* gene expression was performed based on previous evidence of significant increase of the *in vivo* virulence of *P. marinus* exposed to oyster pallial mucus (Palles Espinosa et al., 2013). As a result, the exposure of *P. marinus* to mucus induced significant regulation of nearly 3,600 transcripts, many of which are considered as putative virulence factors (Pales Espinosa et al., 2014).

Proliferation and virulence of *Perkinsus* spp. is modulated by intracellular Fe^{+2}; peroxide antimalarial drugs as iron chelators can inhibit important metabolic pathways of the parasite. Gene expression of Fe^{+2} transport proteins as Nramp, described first in *P. marinus* by Lin et al. (2011), and calcium transport protein (ATP6/SERCA) are highly expressed after exposure to iron chelators showing a way of proliferation control (Araujo et al., 2013).

Genome and transcriptome of *P. marinus* have been sequenced (Joseph et al., 2010); their analysis should provide a better knowledge of the parasite and future prospects in the study of virulence and epidemiology.

I.3.8. Fighting perkinsosis

The fight against perkinsosis is a very difficult battle. Several strategies have been developed in order to minimise the incidence of the parasite, but the eradication is almost impossible in open environment, especially when a disease has been established in an area for a long period (Villalba and Figueras, 2011). The inclusion of *Perkinsus marinus* and *P. olseni* in the list of notifiable diseases of the World Organization for Animal Health (O.I.E.) (http://www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2014/) involves restriction of movements of molluscs from affected areas and other measures that should contribute to avoid disease spreading, especially to non-affected areas. Regarding fighting measures in affected areas, significant advances have been achieved in the case of the infection of oysters *C. virginica* with *P. marinus* but almost no research has been focused on *P. olseni*. Changes in culture procedures and fishery management were addressed to minimise the effects of infection of oysters *C. virginica* with *P. marinus* in Chesapeake Bay (USA), taking advantage of key epidemiological information: (1) salinity influences infection, below a threshold *P. marinus* does not proliferate and, above it, high salinity favours infection (prevalence and infection intensity increase); (2) the influence of temperature determines a seasonal pattern of infection dynamics, with new infections concentrated in the warmest months and mortality peaks in warm months of the next year; (3) infected oysters are the source of new infections, especially when the oyster dies and zoospores become free in the water column (Andrews and Ray, 1988). According to the recommended protocol, oyster producers collected oyster seed from low salinity (non-affected) areas and moved them to high salinity areas for a faster growth; this transfer had to be done in early fall after the highest infective period

(summer) and the harvest should be done before the mortality peak (summer of the second year). All the transferred oysters had to be thoroughly harvested to avoid new infective foci for the next oyster introduction. This protocol worked well for a long period but a successive years of drought (late 1980s) provoked increased salinity in previously non-affected areas, which became colonised by *P. marinus;* since then this fighting procedure lost effectiveness (Villalba and Figueras, 2011). The temperature in the USA Gulf coast is warmer than in Chesapeake Bay, thus seasonality of *P. marinus* infection in the Gulf coast is not as marked as in Chesapeake Bay; management fighting measures are mostly based on salinity regimes (Ray, 1996; La Peyre et al., 2009). Additionally, the accumulation of historical data of infection intensity, mortality and environmental conditions from *Perkinsus* affected areas allowed the development of useful predictive models, which are helping for the management of the *C. virginica* culture and fishery (Hofmann et al., 1995; Powell et al., 1996, 1997; Soniat and Kortright, 1998; Ragone Calvo et al., 2000; Soniat et al., 2006).

Selective breeding programs for oysters *C. virginica* (highly susceptible to *P. marinus* and *Haplosporidium nelsoni* infection) have been developed, with high success at mid-, long-term in some cases, enhancing disease tolerance/resistance and diminishing prevalence and infection intensity (Ford and Haskin, 1987; Gaffney and Bushek, 1996; Ragone Calvo et al., 2003; Abbe et al., 2010; Frank-Lawale et al., 2014). The criterion to select brood-stock for these programmes was very simple and intuitive: survivors with good growth rate under long-term pressure from *P. marinus* and/or *H. nelsoni* were chosen (Abbe et al., 2010; Villalba and Figueras, 2011). Avalilability of molecular markers of tolerance/resistance to select brood-stock should allow more efficient selective breeding programmes, yielding higher survival in shorter term. Different studies of the *C. virginica* genome by amplified length polymorphism (Sokolova et al., 2006), quantitative trait locus (Yu and Guo, 2006) and microarray analysis (Wang et al., 2010) have been developed to search for differentially expressed genes involved in processes such as antimicrobial defence, pathogen recognition, anti-oxidation and apoptosis, which reveal resistance or tolerance to *P. marinus* infection (Wang et al., 2010). Serine protease inhibitor proteins were purified from plasma of *C. virginica*; these proteins inhibit perkinsin, the major extracellular protease of *P. marinus*, as well as other proteases (Xue et al., 2006, 2009). The overexpression of this gene as well as some mutations confers resistance against the parasite due to inhibition of the proliferation of the pathogen (La Peyre et al., 2010; Yu et al., 2011; He et al., 2012).

Another tested strategy to minimise mortality was the use of triploid oysters, which have a faster growth and thus reach market size earlier than diploid ones. Nevertheless, triploid *C. virginica* oysters showed similar susceptibility to *P. marinus* as diploid ones (Barber and Mann, 1991; Meyers et al., 1991).

A more radical strategy to sustain a mollusc industry affected by a devastating disease is the substitution of the susceptible species for a resistant one (Villalba and Figueras, 2011). Two allochthonous species, *Crassostrea gigas* and *Crassostrea ariakensis*, have been tested in the Chesapeake Bay as a remedy against the devastating effects caused by *P. marinus* and *H. nelsoni* in the *C. virginica* industry (Mann et al., 1991; Luckenbach, 2008). However, the introduction of those exotic species into Chesapeake Bay was not advised due to the susceptibility of *C. gigas* to *P. marinus* (Calvo et al., 1999) and *H. nelsoni* (Burreson et al., 2000) and of *C. ariakensis* to *P. marinus* (Calvo et al., 2001; Paynter et al., 2008) and *Bonamia* sp. (Burreson et al., 2004).

The use of therapeutic products to treat diseases of molluscs in the open marine environment is not advisable in open waters but could be successful in in-door culture facilities. Some compounds were tested in the infection of *C. virginica* with *P. marinus* (Calvo and Burreson, 1994; Faisal et al., 1999) and some of them, such as ciclohexamide and bacitracin, cause reduction of the infection level but do not eliminate the parasite completely. Other products inhibit or kill *Perkinsus* spp. *in vitro* (Gauthier and Vasta, 1994; Krantz, 1994; Dungan and Hamilton, 1995; Elandaloussi et al., 2003, 2005a,b; Lund et al., 2005; Panko et al., 2008; Alemán-Resto and Fernández-Robledo, 2014). Some of these substances could be useful in close culture systems for a period of time to prevent infections (Villalba and Figueras, 2011).

I.4. MICROSATELLITES

Microsatellites, also known as simple sequence repeats (SSR) or short tandem repeats (STR), are non-coding repetitive DNA regions composed of small motifs of 1 to 6 nucleotides repeated in tandem, which occur in both eukaryotic and prokaryotic genomes (Field and Wills, 1998; Tóth et al., 2000). Broadly used as genetic markers, microsatellites have a particular attribute in that they suffer higher rates of mutation than the rest of the genome (Jarne and Lagoda, 1996). Microsatellites are classified according to the type of repeat sequence as perfect, imperfect, interrupted or composite. In a perfect microsatellite the repeat sequence is not interrupted by any base not belonging to their motif (e. g. TATATATATATATATA) while in an imperfect microsatellite there is a pair of bases between the repeat motifs that does not match the motif sequence (e. g. TATATATATACTATATA). In the case of an interrupted microsatellite there is a small sequence within the repeat sequence that does not match the motif sequence (e. g. TATATACGTGTATATATA) while in a composed microsatellite the sequence contains two adjacent distinctive sequence-repeats (e. g. TATATATAGTGTGTGTGT) (Oliveira et al., 2006).

Microsatellites have taken advantage over other genetic markers such as amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA

(RAPD) and restriction fragment length polymorphism (RFLP), because microsatellites have the highest rate of polymorphism, are codominants and have a mendelian segregation, the occurrence of a single genetic locus per microsatellite allows a clear observation and easy interpretation of bands in gels, and are selectively neutral (Golstein and Pollok, 1994; Vendramin et al., 1996).

The use of microsatellites is widespread in science with a wide range of applications, such as construction of genetic maps of different types of organisms (Knapik et al., 1998; Cregan et al., 1999), association between the instability of the number of repeats and human genetic diseases (Stallings, 1994; Martin et al., 2009), and studies of population genetics and genotyping and paternity analysis (Borrel et al., 2002; Pardo et al., 2011; Thompson et al., 2011).

Microsatellites can be used to estimate phylogenetic distance among strains or species set in different locations. Microsatellite markers were used to study populations of different parasites includes in Apicomplexa taxa, such as *Plasmodium vivax* (Gunawardena et al., 2010; Van den Eede et al., 2010), *Trypanosoma cruzi* (Llewellyn et al., 2009) and even for *P. marinus* (Thompson et al., 2011)

Studies on genetics of *Perkinsus marinus* populations have been performed using RFLPs (Reece et al., 1997), variation at the ITS and NTS regions of the rRNA gene, and SOD1 and SOD2 genes (Thompson and Hare, 2005), and microsatellites (Thompson et al., 2008; Thompson et al., 2011, Thompson et al., 2014a). These studies showed no evidence for isolation by distance, and even asexual propagation seems the best way of reproduction especially when *Perkinsus* spp. recently arrived; movement of infected oysters may increase out crossing opportunities, potentially facilitating rapid evolution of the parasite (Thompson et al., 2014a). The development of 12 microsatellite markers in *P. olseni* genome (Pardo et al., 2011) provides useful tools for genetic population analysis in this species.

I.5. PROTEOMICS

The term “Proteome” was launched and defined by Wilkins et al. (1996) for the first time. The term was established to obtain an equivalent concept to “genome” and was defined as the full complement of proteins expressed by the genome of one organism, tissue or cell at a specific time. Imperceptibly, the proteome was transmuted into a new discipline, “proteomics”. This new discipline is defined as the high-throughput study of the proteome, including protein quantification, protein-protein interaction, post-translational modifications and protein function (Diz et al., 2012). The proteome is very dynamic, their components vary among organisms, tissues, cells or organelles, and due to changes in their environment, stress, drugs administration, biochemical signals or their physiological or pathological state. All these factors increase the complexity of a proteome due to the activation or

suppression of gene expression, protein interactions, or post-translational modifications (Vázquez-Cobos, 2003). The main objective in proteomics is the identification of these differences and variations. It might be interesting to study the differences at a protein level of samples exposed to different conditions or in a comparison of samples from a healthy and unhealthy organism or tissue (Chicano-Gálvez, 2010). The proteomic field is complementary to genomics in so much as it provides additional information about gene expression and its regulation in different tissues and/or cell types at different times. Nevertheless, proteomics offer information about expressed proteins and post-translational modifications that cannot be deduced from genomics. Studies on the transcriptome or the genome may be insufficient for understanding the phenotype because there is a lack of convergence between the proteome and the transcriptome (Diz et al., 2012).

I.5.1. Proteomic separation tools

In a global view, two main strategies are used to separate proteins:

- Conventional: this strategy involves the separation of all the proteins occurring in a sample by two-dimensional electrophoresis (2-DE), which usually is followed by the identification of proteins with interest by mass spectrometry (MS).

- Shot gun proteomics: It involves the sequencing of a complex mixture of peptides using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The use of shot-gun proteomics allows for a greater number of proteins to be identified rapidly from a single sample, providing a more complete metabolic picture of cellular function and physiology (Schiffman et al., 2013)

I.5.1.1. Conventional proteomics: two dimensional electrophoresis (2-DE) technique

In 1975, Klose, O´Farrell and Scheele published various papers describing high resolution two-dimensional methods. Proteins can be separated in two steps in single spots in a gel according to their isoelectric point (pI) and molecular weight (Mw) (Fig. I.16). This new methods opened the door to the study of a new world of knowledge: the detailed workings of cellular machines (Anderson and Anderson, 1998). This technique can resolve proteins differing in a single charge and consequently, can be used in the analysis of *in vivo* modifications resulting in a change in charge. Proteins whose charge is changed by missense mutations can be identified (O´Farrel, 1975).

Some limitations were found in 2-DE despites this technique has big advantages over other techniques, as the visualisation of all the proteins occurring in a sample and the possible identification of isoform proteins. However, proteins with extreme *pI* (below pH 3 and above pH 10) are very difficult to visualise, hydrophobic membrane proteins are largely absent from samples, and proteins present at less than 1,000

copies per cell are likely undetectable (Görg et al., 2000; Wilkins and Appel, 2007). Another limitation of the technique is the loading capacity and the staining sensitivity of the 2-D gel process. Nevertheless, the combination of 2-DE and MS has become an important analytical technique for the characterisation of complex protein populations extracted from tissue, cell or subcellular fractions. The technique can separate and display more than 10,000 different proteins in a single experiment (Nordhoff et al., 2001).

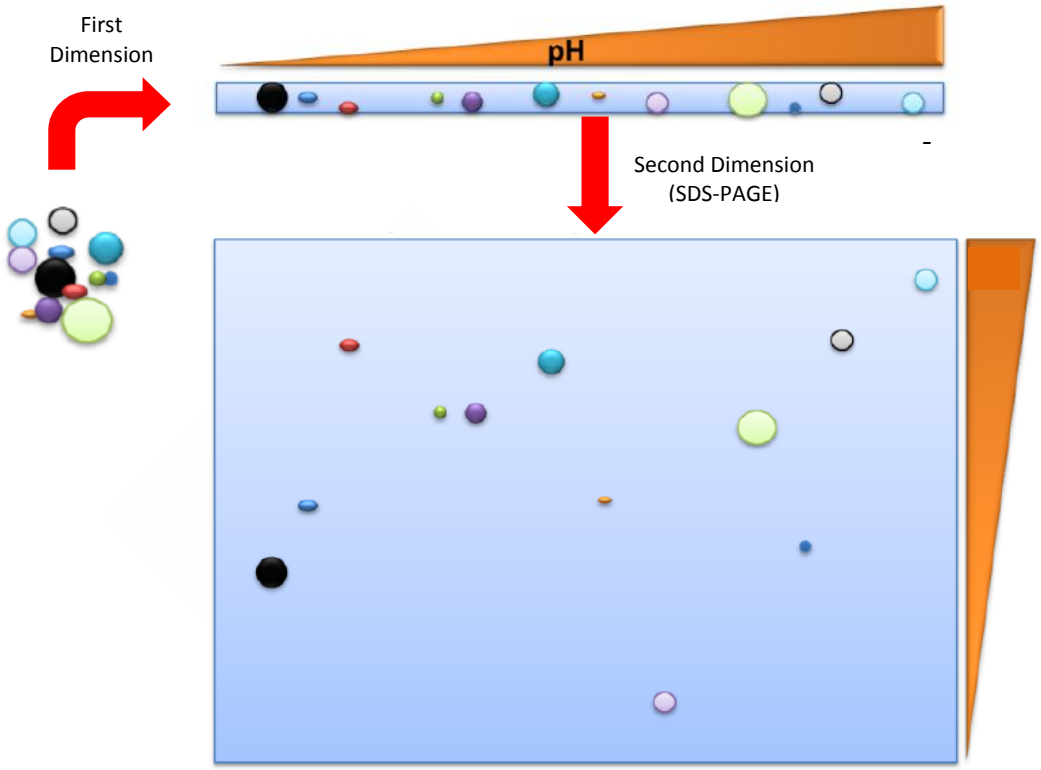

Fig. I.16. General scheme of a two-dimensional electrophoresis. Image taken from Chicano-Gálvez (2010).

I.5.1.1.1. First dimension: isoelectrofocusing (IEF) step

A complex mixture of proteins can be separated by their isoelectric point (pI). The pI of a protein is the pH where its net charge is zero. The introduction of a protein mixture in a gel with pH gradient make that their N and C terminal extremes and their residues catch and release protons according to their pH. When an isoelectric field is applied, all molecules with positive net charge are attracted to the cathode and the molecules with negative net charge to the anode. When the proteins are close to their pI, they lose mobility until their charge is zero and they stop moving. It is in this stage when the proteins are "focused". According to this property of the proteins, immobilised pH Gradient (IPG) strips were developed by Bjellqvist et al. (1982). This pH gradient was created incorporating covalently a gradient of acid and basic buffering groups into a polyacrylamide gel at the time it is cast. These buffering groups are a set of well-characterised molecules, each with a single acidic or basic buffering group

linked to an acrylamide monomer. The general structure is CH_2=CH-(C=O)-NH-R, where R is the weakly acidic or basic buffering group. For improved performance and simplified handling, the IPG gel is cast onto a 3mm wide plastic strips (Berkelman and Stenstedt, 2002). IPG strips with different ranges of pH are commercially available, from wide range strips (3-10 pH) to narrow strips which resolve 1 or 1.5 unit of pH, for example 4-5,5 pH. This offer allows optimising the experiments by choosing the pH range of interest, frequently the range where most proteins are set.

Proteins must be completely disaggregated and fully solubilised in the sample in order to achieve a well-focused first dimension separation. Non-protein impurities in the sample can interfere with separation and subsequent visualisation of the 2-DE result, thus sample preparation can include steps to rid the sample of these substances. Salts, nucleotides, phospholipids, polysaccharides, lipids, phenolic compounds and insoluble material are the major problems for a correct visualisation. In marine samples salts should be removed or maintained at as low a concentration as possible to avoid streaking or problems in focusing process (Berkelman and Stenstedt, 2002). Thus, detergents and high concentration of urea are used to ensure a complete solubilisation and denaturation. IEF performed under denaturing conditions gives the highest resolution and the cleanest results. In the first dimension, urea is widely used as denaturant. It is always included in the protein solubilisation buffer at a concentration of at least 8 M. Urea solubilises and unfolds most proteins to their fully random conformation, with all ionisable groups exposed to solution. The use of thiourea in addition to urea improves the solubilisation of the sample, particularly of membrane proteins (Rabilloud, 1998). A non-ionic (uncharged) or zwitterionic (having both positively and negatively charged groups, but with a net charge zero) detergents are always included in the solubilisation buffer to help solubilise the hydrophobic residues that are exposed as a result of denaturation of chaotropes (urea and thiourea). Traditionally, some detergents as NP-40 or Triton X-100 have been used; more recently they have been superseded by the 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (Perdew et al., 1983; Berkelmand and Stendstedt, 2002). Reducing agents are included in the sample solution to break any disulfure bonds and to maintain all proteins in their fully reduced state. The most commonly used reductant is dithiothreitol (DTT). Carrier ampholytes or IPG buffer can be included in the sample solution to enhance protein solubility by minimizing protein aggregation due to charge-charge interactions (Berkelman and Stenstedt, 2002).

I.5.1.1.2. Second dimension: sodium dodecyl sulfate – polyacrilamide gel electrophoresis (SDS-PAGE)

For the second dimension, IPG strip is set on a polyacrilamide gel and a common electrophoresis is performed. In this step, proteins are separated according their molecular weight (Mw). This technique is performed in polyacrylamide gels

containing sodium dodecyl sulfate (SDS). SDS is an anionic detergent that denatures proteins by wrapping around the polypeptide backbone. The SDS also disrupts hydrogen bonds, blocks hydrophobic interactions and partially unfolds the protein molecules, minimising differences in molecular form by eliminating the tertiary and secondary structures (Berkelman and Stenstedt, 2002).

I.5.1.1.3. Staining procedures

The choice of the staining procedure is critical. Some of them are more sensitive than others, some procedures support quantitative analysis and other do not, only some of them are compatible with posterior MS sequencing and the cost of some of them could be a limitation. Several staining methods to visualise the spots in the gels produced by SDS-PAGE have been developed; silver staining, Commassie blue and fluorescent Sypro Ruby are the most used staining procedures, with high sensitivity, but only Commassie blue and Sypro Ruby allow quantitative analysis (with them, staining intensity is proportional to protein concentration in the spot) (Patton, 2000).

The most sensitive staining is silver staining. The detection limit is as low as 0.1 ng of protein per spot, but it has some disadvantages such as the poor reproducibility, limited dynamic range, and it is a non-linear stain, which does not allow to properly quantify the concentration of protein in each spot (Görg et al., 2000). Coomassie staining is a quantitative method but it is 50-fold less sensitive than silver stain. Some modifications of the Coomassie Blue have led to Coommassie Blue G-250, with sensitivity closer to that of silver staining. The resolution of this new Coommassie Blue G-250 staining procedure is up to 1 ng of protein per spot (Candiano et al., 2004). Several flourescent stains have been developed in the last years. The SYPRO family, such as SYPRO red, SYPRO orange or SYPRO tangerine, have been reported to interact with SDS-protein complexes with sensitive levels in the order of 1-4 ng of protein per spot. In the SYPRO family, the most used staining method is the SYPRO Ruby stain, due to its high sensitivity and simplified handling. The detection sensitivity of SYPRO Ruby is 0.5-1 ng per spot; quantification of protein concentration is possible. One possible limitation of this staining method is the cost of the equipment for fluorescence image acquisition; fluorescence emission of the dye is approximately 618 nm and robust and expensive scanners are necessary (Görg et al., 2000; Candiano et al., 2004).

The gel images can be compared with different commercial softwares, such as PD Quest (BioRad Laboratories), Proteamweaver (BioRad Laboratories), DeCyder (GE Healthcare), SameSpots (Nonlinear Dynamics), Melanie (Geneva Bioinformatics) or specific services as Ludesi (http://www.ludesi.com).

I.5.1.1.4. Two-dimensional differential gel electrophoreis (2D-DIGE)

Nowadays, more than one sample can be compared in the same gel using molecular labels. This new technology is called Differential Gel Electrophoresis (DIGE)

(Tonge et al., 2001) (Fig. I.17). 2D-DIGE is based on the properties of 3 fluorescent dyes of the cyanine family (CyDye: Cy3, Cy2, Cy5); each dye has differential fluorescence read-out. Each sample is labelled with one dye, mixed and focused together in the IEF, thus three samples are present in each IPG strip (2 problem samples and 1 control, which consists of the mixture of the two problem samples in order to get one internal control). The technique allows the comparison of samples with the advantage of avoiding variation between gels but it shares limitations with classical 2-DE regarding extremes of pI, Mw range and hydrophobic membrane proteins (Kussman et al., 2006).

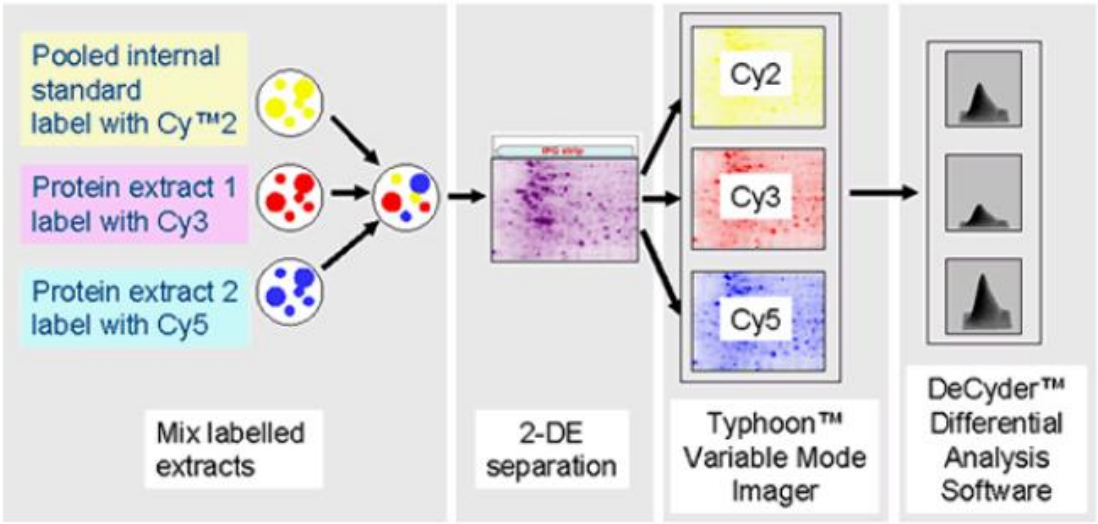

Fig. I.17. Scheme of Differential Gel Electrophoresis (2D-DIGE). Image taken from www.amershanbiosciences.com

I.5.1.2. Shotgun proteomics

Shotgun proteomics is a conceptually different strategy emerged for protein analysis in proteomics. It involves taking complex mixtures of proteins or indeed a whole proteome, and digest all proteins to peptides with endoproteinases of known specificity. The resulting mixture of peptides is then analysed using liquid chromatography and tandem mass spectrometry (LC-MS/MS). Peptide fragment data are matched against sequence databases to determine the proteins present in a sample (Wilkins and Appel, 2007).

Shotgun proteomics is a very dynamic strategy; different designs are possible depending on researcher needs and sample properties:

- Label approach: Isotope-coded Affinity tag (ICAT) or Isobaric tags for relative and absolute quantitation (iTRAQ) are techniques where samples are labelled with different dyes and pooled together to be analysed by LC-MS/MS (Silberring and Ciborowski, 2010). Stable isotope labelling by amino acid in culture (SILAC) is a

technique based on mass spectrometry that detects differences in protein abundance among samples using non-radioactive isotopic labeling (Everley et al., 2004).

- Label free: is often used to study the whole proteome of a sample or specie. All proteins are sequenced by LC-MS/MS allowing the study of several replicates without the over cost of labeling (Schiffman et al., 2013).

The choice of the technique is a critical step in an experiment because several different ways can be developed (Fig. I.18).

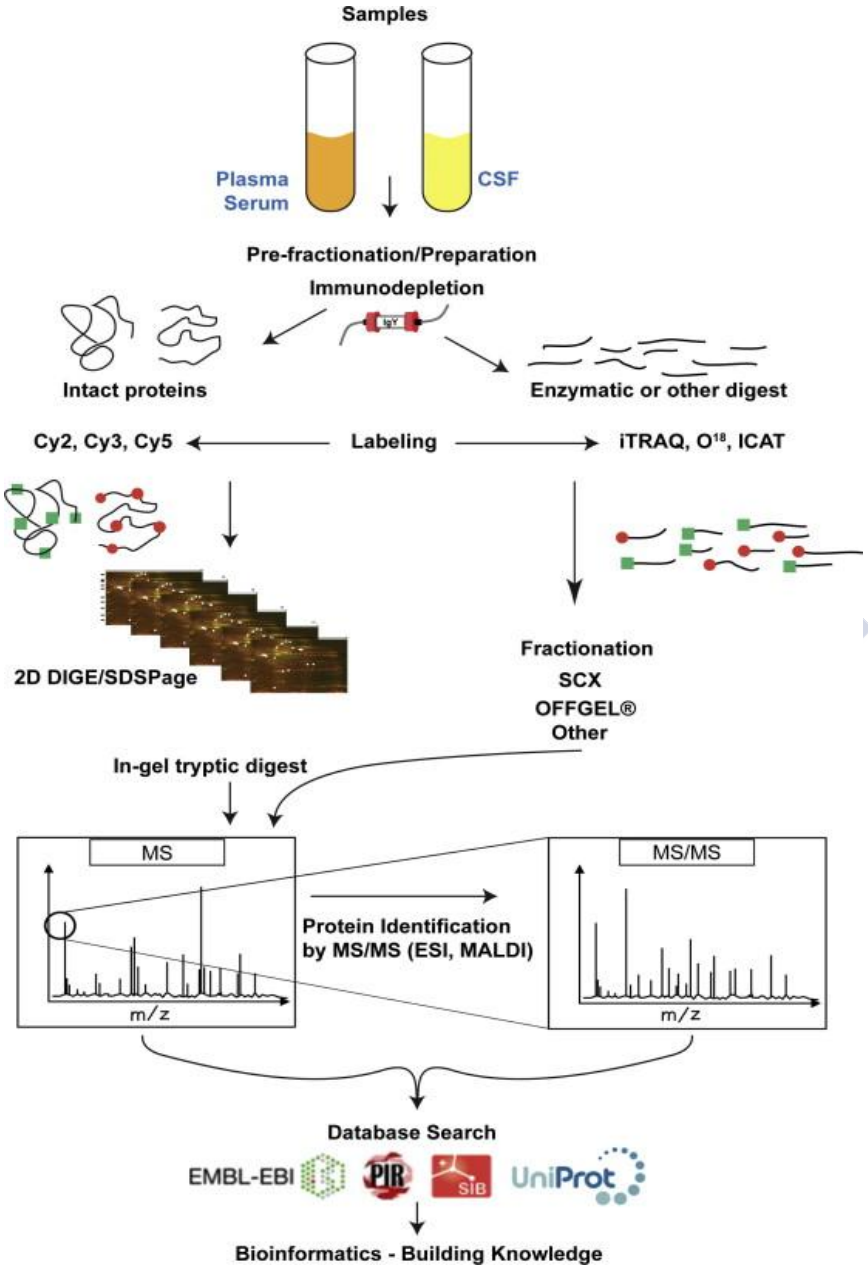

Fig. I.18. Typical proteomic workflow used in clinical proteomics leading to biomarker discovery. Image taken from Silberring and Ciborowski (2010).

I.5.2. Mass spectrometry

Mass spectrometry characteristics have raised it to an outstanding among analytical methods: unequalled sensitivity, detection limits, speed and diversity of its applications. In analytical chemistry, the most recent applications are mostly oriented towards biochemical problems, such as proteome, metabolome, high throughput in drug discovery and metabolism and so on. Other analytical applications are routinely applied in pollution control, food control, forensic science, natural products or process monitoring (de Hoffman, 2007).

Mass spectrometry has progressed extremely rapidly during the last decade. This progress has led to the advent of entirely new instruments. New atmospheric pressure devices were developed (Robb et al., 2000; Laiko et al., 2000; Takats et al., 2004, Cody et al., 2005), existing analysers were optimised and new hybrid instruments were produced by new combinations of analysers.

A mass spectrometer always contains the following elements: a sample inlet to introduce the compound that is analysed; an ionisation source to produce ions from the sample; one or several mass analysers to separate the various ions; a detector to "count" the ions emerging from the analysers; and finally a data processing system that produces the mass spectrum in a suitable form. However, some mass spectrometers combine the sample inlet and de ionisation source and others combine the mass analyser and the detector.

A mass spectrometer should always perform the following process: I) produce ions from the sample in the ionisation source; II) separate these ions according to their mass-to-charge ratio in the mass analyser; III) eventually, fragment the selected ions and analyse the fragments in a second analyser; IV) detect the ions emerging from the last analyser and measure their abundance with the detector that converts the ions into electrical signals; V) process the signals from the detector that are transmitted to the computer and control the instrument through feedback (de Hoffman and Stroobant, 2007).

I.5.2.1. Ionisation

In order to be analysed in a mass spectrometer, the sample must be ionised and it is imperative that the ionised molecules (proteins or peptides) turn into a gas phase to allow analysis, fragmentation and detection (Wilm, 2011). Several ionisation methods exist, but the most commonly used methods in proteomics are matrix-assisted laser desorption and ionisation (MALDI) and electrospray ionisation (ESI) (Hernández et al., 2007).

I.5.2.1.1. Matrix-assisted laser desorption ionisation (MALDI)

For MALDY analysis, analytes are first embedded into a crystalline matrix on a metal "target" plate. When this target is placed into the vacuum of a MALDI source, pulses of laser light are directed to the matrix, causing vibrational excitation and ejection of the analyte molecules and the matrix components. As the matrix evaporates, analytes are liberated and ionised (Fig. I.19). Generally, the observed ions of proteins and peptides are protonated and carry a single charge (Hernández et al., 2007).

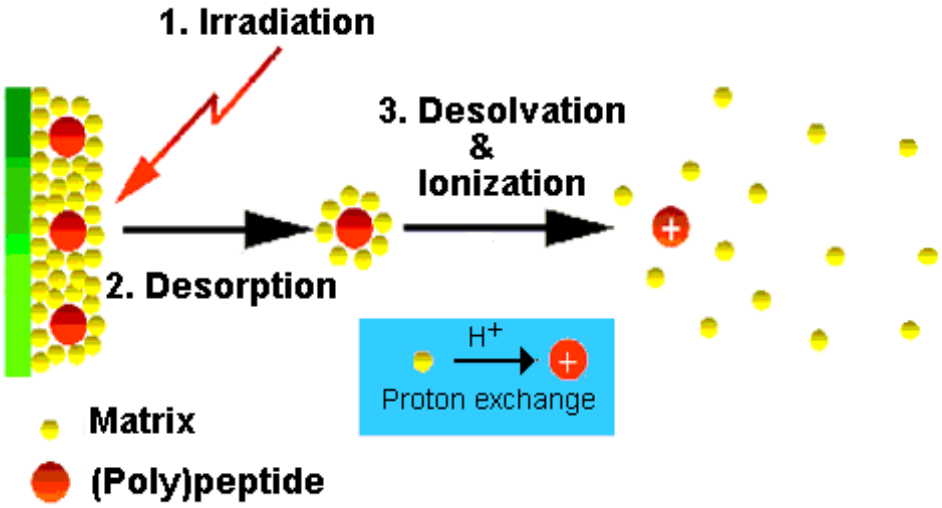

Fig. I.19. Scheme of an ionisation source in MALDI procedure. Image taken from www.ru.nl /science/gi/facilities/other-devices/maldi-tof/

I.5.2.1.2. Electrospray ionisation (ESI)

In an ESI source (Fig. I.20), the sample is presented to the mass spectrometer in a liquid form at atmospheric pressure. It flows into a needle that is subject to high voltage. The electrical potential applied between the needle tip and the inlet of the mass spectrometer leads to an accumulation of the same type of charges on drops that exit the needle tip. These solvent drops spontaneously dissociate to form a fine spray of highly charged droplets due to electrostatic repulsion. The flow of droplets is directed through a countercurrent flow of heated gas, causing the solvent to evaporate and the droplets to shrink. This causes increment of the charge concentration on the surface of the droplets. As the electrical charge reaches a critical state, the droplets explode into smaller and lower charged particles. This process of shrinking and explosion is repeated until no individually charged analyte molecules remain. Generally, a mixture of singly and multiply charged ions is generated. Since the sample is introduced in a liquid state at atmospheric pressure, ESI sources can easily be associated with online liquid-phase separation techniques, such as liquid chromatography (Hernández et al., 2007).

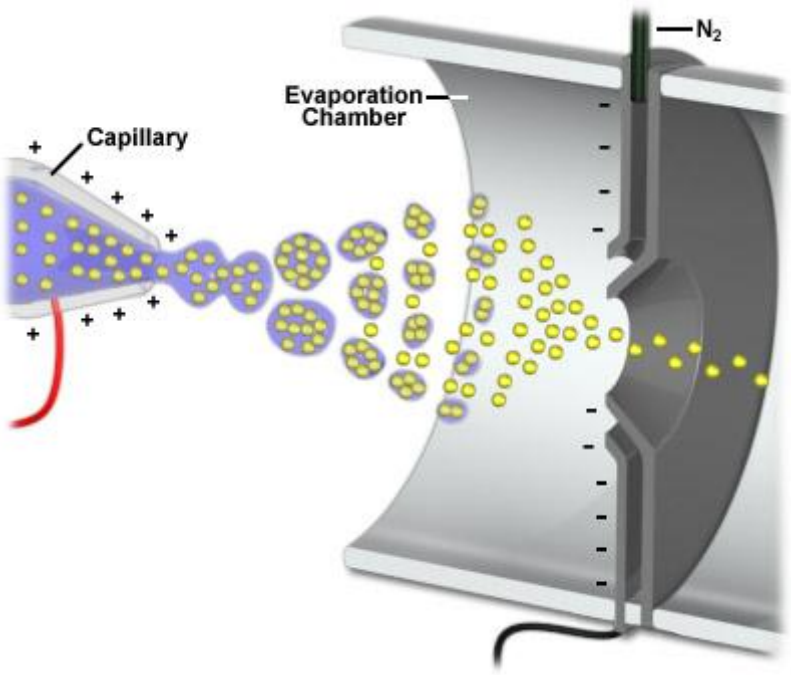

Fig. I.20. Scheme of Electrospray Ionization process. Image taken from www.magnet.fsu.edu

I.5.2.2 Analysers

Once the sample is ionised, it enters the mass spectrometer itself. The mass analysers explore different characteristics of the parent or fragment ions. In case of tandem experiments, the parent ion must be selected for further fragmentation, and the generated fragment ions will be detected. The combination of two or more analysers in the same mass spectrometer yields the high performance and resolution of the nowadays equipments. The main function of a mass analysers is to separate the ions according to their m/z ratio (March, 2009), basically by their behavior in electric or magnetic fields (El-Aneed et al., 2009). There are few types of analysers used in proteomic research: time-of-flight (TOF), quadrupole ion trap, orbitrap and Fourier Transform FT ion cyclotron resonance (ICR) analysers (Hernandez et al., 2007).

I.5.2.2.1. Time of flight analyser

TOF mass analysers measure ions that are accelerated in an electric field, then travel down a field-free vacuum tube towards an ion detector (Fig. I.21). All ions in the source are given the same amount of kinetic energy, but their velocity is a function of their mass and charge. The time needed to travel the distance between the source and the detector is therefore dependent on their m/z values. This can be calculated using the kinetic energy equation, given the tube length and the measured times of flight (Hernández et al., 2007).

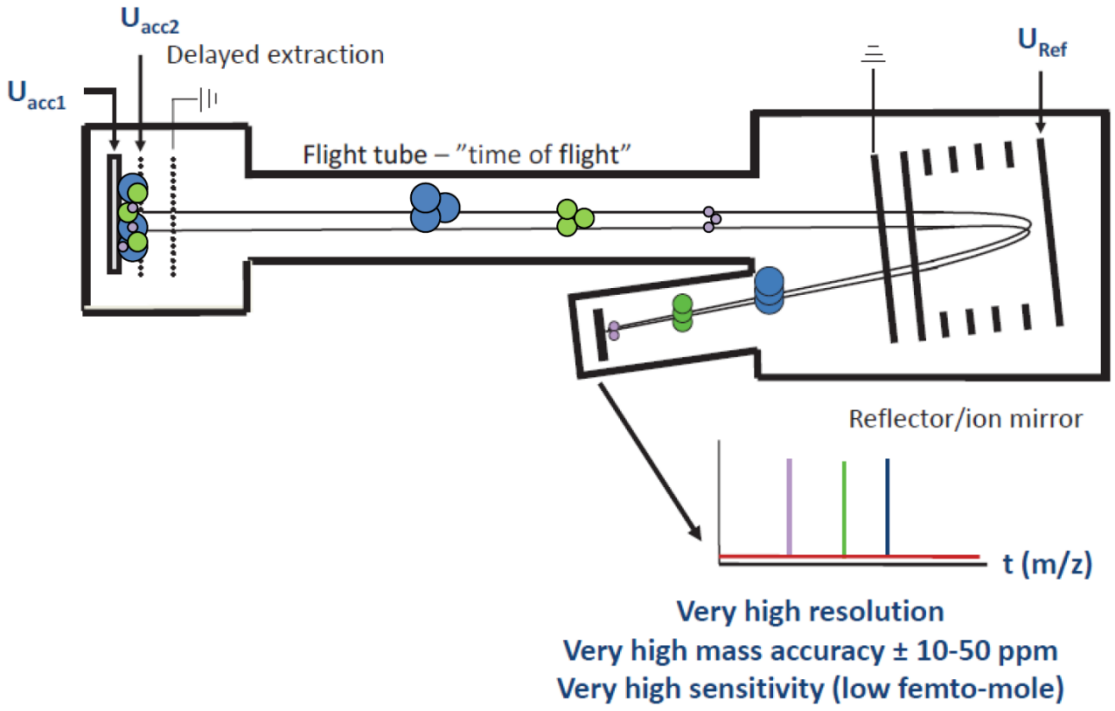

Fig. I.21. General scheme of a MALDI TOF MS. Image taken from Hjernø and Højrup (2012).

I.5.2.2.2. Quadrupole

Quadrupole mass analysers (Fig. I.22) consist of four parallel and symmetrically arranged metallic roads. One couple of opposite rods have a positive electrical potential, while the other couple of opposite rods have a negative electrical potential. Ions oscillate while traversing the field along the central axis of the rods. Depending on the voltages applied, ions are either ejected from the quadrupole or sent to the detector, therefore the quadrupole is considered as mass filter. To obtain a complete spectrum, one couple continuously varies or scans the electromagnetic field in the quadrupole while the sample passes through the analyser (Hernández et al., 2007).

I.5.2.2.3. Ion trap

Ion trap mass analysers are devices that can store or trap charged molecules for long time. Ions are trapped by electric potentials produced by a ring-shaped electrode and two end-cap electrodes. This occurs in a space of 2-3 cm^3 that is filled with an inert gas. Ions of different m/z values enter the trap at one of the end-cap electrodes and remain trapped, oscillating at frequencies that are related to their m/z values. The ions are then subjected to additional electric fields, which eject one ion species after another from the trap and they are detected, to produce a mass spectrum (Mann et al., 2001; Hernández et al., 2007).

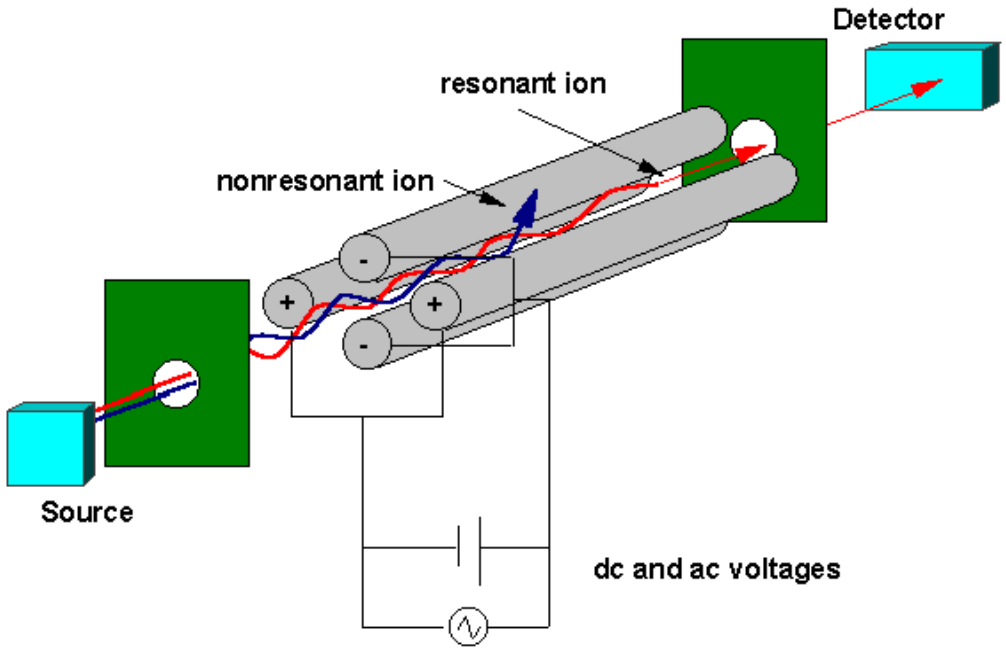

Fig. I.22. Schematic representation of a quadrupole in a mass spectrometer. Image taken from www.files.chem.vt.edu.

I.5.2.2.4. FT-ICR

FT-ICR mass analysers allow ions to be accumulated and stored for periods as long as minutes. FT mass spectrometers consist of a cubic cell inside a strong magnetic field. Injected ions rotate around the magnetic field with a frequency typical for their m/z. By varying the electric fields, changes in the ion frequency of rotation can be measured and converted into m/z using a Fourier transformation (Hernández et al., 2007).

I.5.2.3. Fragmentation

There is no doubt that the fragmentation step of a precursor ion is a key point in proteomics analyses since it enables analyses at the tandem mass spectrometry (MS/MS) levels. In MS/MS analyses, the first analyser selects the ion(s) which proceeds to a subsequent section, where the excitation and dissociation steps will happen. Tandem mass spectrometry analyses are the result of two or more sequential separations of ions usually coupling two or more mass analysers (Glish et al., 2003; El-Aneed et al., 2009). The most common fragmentation methods used in proteomics are: collision induced dissociation (CID) (Hadden et al., 1968), electron capture dissociation (ECD) (Zubarev et al., 1998) and electron transfer dissociation (ETD) (Syka et al., 2004).

I.5.2.4. Detectors

The ions pass through the mass analyser and are then detected and transformed into an usable signal by a detector. Detectors are able to generate an electric current from the incident ions, which is proportional to their abundance. There are several types of detectors. The choice of detector depends on the design of the instrument and the analytical applications that will be performed. A variety of approaches are used to detect ions. However, detection of ions is always based on their charge, their mass or their velocity. Some detectors are based on the measurement of direct charge current that is produced when an ion hits a surface and is neutralised. Others are based on the kinetic energy transfer of incident ions by collision with a surface that in turn generates secondary electrons, which are further amplified to give an electronic current (de Hoffman and Stroobant, 2007).

I.5.2.5. Computers

A computer dedicated to mass spectrometry is usually capable of three basic operations: I) control of the mass spectrometer; II) acquisition and processing of data from the mass spectrometer; and III) interpretation of data. The computer can control the mass spectrometer by introducing the values and variations of different parameters. Because the computer processes digital data, whereas the mass spectrometer produces and receives analogic data, an interface is necessary to convert one type of data in another. A computer dedicated to mass spectrometry records the data given out by the mass spectrometer and converts them either into values of masses and peak intensities, or into total ionic current, temperatures, acceleration potential values, and so on. It is also capable of data processing. It allows calculation of average spectra and subtraction of one spectrum from another in order to eliminate the background noise or simply emphasise the differences between two spectra (de Hoffman and Stroobant, 2007).

I.5.3. Protein identification

There are two major methods that are widely used for protein identification by mass spectrometry (MS): Peptide mass fingerprinting and Tandem MS.

I.5.3.1. Peptide mass fingerprinting

Peptide mass fingerprinting (PMF) is an analytical technique for protein identification in which proteins are first digested using a site-specific proteolytic enzyme. The masses of the resulting peptides are then determined by MS. Since each protein has a different sequence, the masses obtained for each protein are an unique "fingerprint". Protein identification is performed by comparing the experimentally determined peptide masses with theoretically determined peptide masses generated from protein sequences in databases by means of mass search programmes. Scoring

systems are used to rank proteins, whereby the high-ranking proteins from the database have the largest numbers of peptides in common with the protein that has been analysed. Scoring systems for PMF are critical, and should take into account many factors to produce a robust score. These factors include dissimilarities in the peptide masses due to calibration errors, expected peak intensities, noise, contaminant or missing peaks, presence of post-translational modifications… (Hernández et al., 2007).

The PMF approach is rapid and efficient, but it has some limitations when:

- Samples contain a mixture of proteins. The complexity of such spectra can result in false positives identifications.

- MS spectra are searched against large sequence databases. As the specificity of the method is based on statistics, the larger database, the higher number of randomly matched peptide masses.

- The proteins carry unexpected modifications reducing the number of matching peptide masses.

- The proteins under analysis are very small or very large. Very small proteins produce very small number of peptides to be analysed. It is possible that these few peptides might not be present in a MS spectrum. In the case of large proteins, the number of theoretical peptides is so large that a portion of them are likely to randomly match nearly every spectrum.

- The protein sequence under investigation is not represented in the protein sequence database (Hernández et al., 2007).

I.5.3.2. Tandem mass spectrometry

Protein identification from tandem mass spectra is one of the most versatile and widely used proteomics workflows, able to identify proteins, characterise post-translational modifications, and provide semi-quantitative measurements of relative protein abundance (Edwars, 2011). MS/MS is widely used as an alternative to PMF. As a number of peptides are usually fragmented for each protein, the identification is more robust and less equivocal; it is more robust because there is no need to identify all peptides of a given protein to achieve confident identification; it is less equivocal because the identification of several peptides for a given protein confirms its presence in the sample (Hernández et al., 2007). Tandem mass spectra contain structural information related to the sequence of the peptide, rather than only its mass; these searches are generally more specific and discriminating (Mann et al., 2001; Chicano-Gálvez, 2010).

I.5.3.2.1. The peptide fragment fingerprinting approach

The principle of protein identification by peptide fragment fingerprinting (PFF) is similar to that of PMF. The aim is to correlate an experimental MS/MS spectrum with virtual MS/MS spectra constructed from the theoretical digestion of proteins to peptides and fragmentation of its peptides. A matching score is calculated that depends on the correlation between the experimental spectrum and the virtual spectrum of the peptide being compared (Hernández et al., 2007). The main advantages of this method are the higher discrimination capacity and the analysis of proteins mixtures allowing the use of massive sequencing techniques (Chicano-Gálvez, 2010). The computational analysis typically starts with the identification of the peptides that give rise to de acquired MS/MS spectra. In high-throughput studies, the most efficient peptide identification method is based on searching MS/MS spectra against protein sequence databases such as Sequest and Mascot (Nesvizhskii, 2007).

I.5.3.2.2. De Novo *Sequencing*

The growth of new sequenced species is simplifying the task of determining the primary structures of peptides and proteins, because open reading frames in the nucleotide sequence serve as templates for the construction of the corresponding proteins (Standing, 2003). The masses of the peptides produced by proteolytic digestion of an unknown protein can be compared with those predicted to arise from each protein in the database. This is often sufficient to identify a protein whose full-length sequence is contained therein. But if the genome sequence of an organism is still unknown or the identification through PMF, or PFF does not work, the complete characterisation of the protein primary structure or *de novo* sequencing is required (Mann et al., 2001; Aebersold and Mann, 2003). *De novo* sequencing requires tandem mass spectrometry (MS/MS) in order to determine the order of the aminoacid sequences in a peptide. This includes accurate measurements of low mass ions, such as immonium ions. In this technique, a given parent (precursor) ion is selected in one mass spectrometer and then broken up, usually by collisions. The m/z values for the resulting daughter (product) ions are measured in a second mass spectrometer. The advantage of the *de novo* sequencing approach over the database search method is that it allows identification of peptides whose exact sequence is not present in the searched sequence database. However, *de novo* analysis is computationally intensive and requires high-quality MS/MS spectra. Peptide sequences extracted from MS/MS spectra using *de novo* algorithms need to be matched against the sequences of known proteins present in the sequence databases. In the case of organisms with non-sequenced or only partially sequenced genomes, the database search approach will fail to assign correct peptide sequences to many MS/MS spectra, thus using *de novo* sequencing tools becomes necessary (Liska and Shevchenko, 2003).

I.5.4. Sequence database searching

For some organisms, multiple sequence databases are available (Apweiler et al., 2004). The most common used databases are: the National Center for Biotechnology Information (NCBI) Entrez Protein database, the NCBI Reference Sequence (RefSeq) database, that provides a non-redundant collection of sequences representing genomic data, transcripts and proteins (Pruitt et al., 2005), and The Universal Protein Source (UniProt) (consisting of Swiss-Prot, which is manually annotated and reviewed and its supplement TrEMBL, which is automatically annotated and is not reviewed) (Nesvizhskii, 2007).

Sequence alignment has become the central tool for sequence comparison in molecular biology. In bioinformatics, a sequence alignment is a way of arranging the primary sequences of DNA, RNA or protein to identify regions of similarity that may be a consequence of functional, structural or evolutionary relationships between the sequences (Lesk, 2002). Aligned sequences of nucleotide or amino acid residues with identical or similar characters are aligned in successive columns. Very short of similar sequences can be aligned by hand. Most interesting problems require the alignment of lengthy, highly variable or extremely numerous sequences that cannot be aligned solely by human effort. Instead, human knowledge is primarily applied in constructing algorithms to produce high-quality sequence alignments, and occasionally, in adjusting the final results to reflect patterns that are difficult to represent algorithmically. A lot of computational algorithms, such as Basic Local Alignment Search Tool (BLAST) and Fast Alignment (FASTA) have been applied to sequence alignment problem, including slow but formally optimising methods like dynamic programming or probabilistic methods designed for large-scale database searches. Due to the high speed, sensitivity and the current availability of up-to-date sequence database, BLAST is the most widely used computer programme for database searches that has been developed; on the contrary, FASTA is much lower (Henikoff and Henikoff, 1992) but it is more suitable for nucleic acid sequence searches (Oladede et al., 2009).

Data base searching is an essential element of large-scale proteomics. Eng et al. (1994) presented their search engine Sequest; the approach of this programme is based on cross-correlation between the fragment ion spectrum and the predicted list of m/z values of predicted ions for each potential peptide (Sadygov et al., 2004). Since then, several search engines have been developed based on Sequest software like the MS/MS-based search engine Mascot (www.matrixscience.com) which assigns a probability-based score and an expectation value to each search hit (Creasy and Cotrell, 2002). Three main types of sequence databases are suitable for searching mass spectrometric data. Non-redundant protein databases (nrdb) contain the known set of full-length protein sequences, extracted from the major sequence repositories and purged of duplicates. The nrdb maintained at the European Bioinformatics Institute

(EBI) allows consulting more than one hundred different databases and have several analysis tools of biological information as Blast and Fasta. The most popular protein databases are SwissProt and Uniprot Knowledgebase together with TrEMBL; they are accessible all together in the European Bioinformatics Institute website (www.ebi.ac.uk /Tools/sss/ncbiblast/) or in Uniprot (www.uniprot.org). The main server of SwissProt and TrEMBL is Expasy (www.expasy.ch), it is specialised in proteins and offers a high number of tools for sequence, structure and function analysis. Expressed sequence tag (EST) databases, such as dbEST at the National Center for Biotechnology Information (NCBI), contain millions of short one-pass sequences from random sequencing of cDNA libraries. These can be searched with appropriate software, usually by translating into the open reading frames. Genome databases can also be searched with MS data. The advantages of searching databases of completely sequenced genomes are that each peptide must be present by definition and that often the MS data can help to define the structure of the gene, such as start and stop and intron-exon structure (Mann et al., 2001).

I.5.5. Proteomic applications for molluscs and parasites

Proteomic analysis could provide an integrated "snapshot" of the functional proteins that can change in the course of all kinds of biological events, such as development, evolution and pathogenicity (Chen et al., 2011). This technology has been used in the study of molluscs for several applications, from ecological adaptation to search for biomarkers in contamination events or disease infection. López et al. (2001) detected differences in proteins expression between intertidal and cultured mussels, the intertidal mussels expressed more heat shock proteins than those cultured from rafts. Fuentes et al. (2002) found a lower viability of hybrid mussels between *Mytilus edulis* and *M. galloprovincialis* than "pure populations"; the lower viability was associated with higher parasitisation and lower expression level of stress proteins.

Biomonitoring of aquatic environment and assessment of ecosystem health play essential roles in the development of effective strategies for the protection of the environment, human health and sustainable development. The growing interest in the application of proteomic technologies to solve toxicological issues and its relevance in the search for proteins involved in toxicological responses as biomarkes makes the proteomics a highly widespread tool (Monsinjon and Knigge, 2007; Lemos et al., 2010; Campos et al., 2012). Using heavy metals as indicator of pollution, changes in protein expression between clean and polluted areas were found. These changes disappeared when metal content was normalized, thus demonstrating that proteomics is an excellent tool for the search of biomarkers in invertebrates (Rodriguez-Ortega et al., 2003; Romero-Ruiz et al., 2006). Effects of Cadmium, an environmental stressor due to its toxicity, were measured by 2-DE in tissues of *R. decussatus*, the results showed a

diminution in protein expression of the affected tissues, especially in proteins involved in cytoskeletal maintenance (Chora et al., 2009). The level expression of heat shock proteins (hsp 60, 70 and 90 kDa) in response to the contaminant p,p´-dichlorodiphenyldichloroethylene (DDE) was studied in tissues of *R. decussatus* by immunobloting. The results showed a tissue-specific formation of reactive oxygen species in clams (Dowling et al., 2005). As well as in clams, toxicological effects were measured by 2-DE in abalones *Haliotis diversicolor supertexta* exposed to the endocrine disruptor bisphenol-A (Zhou et al., 2010), and in oysters *Crassostrea angulata* following the bioaccumulation of Hg in the food chain, suggesting that protemics would be further developed in application research of food safety (Zhang et al., 2013).

The effects on marine invertebrates of toxic molecules produced by dinoflagellates and other microalgae can be evaluated by proteomic approaches. During seasonal harmful algal blooms many filter-feeding invertebrates can accumulate phycotoxins at extremely high levels representing a serious threat to human health. Proteomic analysis can be used for the detection and identification of biomarkers of biotoxin contamination and components that participate in the tissue response to the exogenous contaminant (Ronziti et al., 2008; Manfrin et al., 2012).

As in human diseases, proteomics was used to detect mollusc proteins expressed in the host-parasite interaction, in order to identify new proteomic markers of disease resistance. That is the case of superoxide dismutase-like molecules from the oyster *Saccostrea glomerata* in the infection with the protozoan parasite *Marteilia sydneyi*, the causative agent of QX disease (Simonian et al., 2009a, b). Cao et al. (2009b) found differences in haemolymph protein expression between *Ostrea edulis* infected and non-infected with the protozoan parasite *Bonamia ostreae*; differences in the protein expression between *Ostrea edulis* (susceptible to infection with *B. ostreae*) and *Crassostrea gigas* (resistant to infection) were also recorded. Viral necrosis infection in *Chlamys farrery* was also studied by 2DE; the immune response of haemocytes against this infection was analysed and 48 proteins associated with immune response were reported (Chen et al., 2011). More recently, a similar approach developed by Castellanos-Martínez et al. (2014) addressed the immune response of the cephalopod *Octopus vulgaris* against the coccidian parasite *Aggregata octopiana*. Several proteins related to immune response were identified both in haemocytes and plasma. These kind of studies are particularly useful for a better comprehension of immune response of the host against parasites, especially in non-model species providing information for subsequent studies. Proteomics have been used to reveal protein expression of molluscs after challenge with bacteria as well. The scallop *Chlamys farreri* showed a differential expression of 27 proteins, most of them related with immune expression, after challenge with *Vibrio harveyi* (Huan et al., 2011). In mussels, the response after induction with *Micrococcus luteus* and *Vibrio anguillarum*

was measured in gills (Ji et al., 2013) and hepatopancreas (Wu et al., 2013), showing a differential protein expression after exposition with bacteria; *Vibrio anguillarum* provoked an increase of oxidative stress and disturbance in energy metabolism while *M. luteus* caused immune stress and disturbances in signaling pathways and protein synthesis.

In the case of parasites theirselves, the study of protein expression of several parasites of the Apicomplexa group have been developed by 2DE. Proteomics analysis of the life cycle could help to settle basis for understanding intracellular and extracellular survival, invasion of host cell membranes, and evasion of host immune systems (Florens et al., 2002; Lasonder et al., 2002; Bautista et al., 2014). Proteomic maps of parasites such as *Toxoplasma gondii* (Cohen et al., 2002), *Neospora caninum* (Regidor-Cerrillo et al., 2012), *Plasmodium falciparum* (Lasonder et al., 2002; Vincensini et al., 2005) and *Leishmania* spp. (Drummelsmith et al., 2003; Walker et al., 2006; Cuervo et al., 2007; Vergnes et al., 2007) have been developed, some of them in different stages of the life cycle of the parasite, in order to get a snapshot of the parasite infection progress (Florens et al., 2002). Studies comparing species of the same genus or attenuated vs virulent strains allow the search for potential targets for drug design and provide a better knowledge of mechanisms associated with virulence.

I.5.6. Functional genomics and proteomics

The term proteome was first coined to describe the set of proteins encoded by the genome (Wilkins et al., 1996). The proteome is much more dynamic than the genome. Likewise, the term “proteome” led to a new research field called proteomics, defined as the high-throughput study of the proteome, including protein quantification, protein-protein interactions, post-translation modifications (PTMs) and protein function (Jensen, 2006; Schrimpf et al., 2009). The PMTs affect the structure, locations, function, and exchange, and affect activation and regulation functions, response to the environment, etc., and also are critical for control of the protein degradation processes (Gygi, 1999). Therefore, many proteins are present in multiple molecular forms. This essential information can only be determined by studying proteins and not genes. Besides, there is no direct correlation between the levels of RNA expression (transcription) and protein expression (Gygi, 1999). A short half-life messenger RNA (mRNA) can generate a long half-life protein, and vice versa. When the protein is present in the cell (and can be detected) the mRNA already cannot be detected. The expression of mRNA does not reveal the activity of the protein that it encodes or its possible combinations or interactions with other proteins that generate new functions, etc (Abbott, 1999). Post-translational modifications generate tremendous diversity, complexity and heterogeneity of gene product and their determination is one of the main challenges in proteomics research. Recent developments in mass spectrometry-based approaches for systematic, qualitative and

quantitative determination of modified proteins promise to bring new insights on the dynamics and spatio-temporal control of protein activities by post-translational modifications, and reveal their roles in biological processes and pathogenic conditions (Jensen, 2004; Kucharova and Wiker, 2014).

The publication of the human and other genomes has demonstrated some key methodological advances that have contributed to the development of many disciplines such as taxonomy, phylogenetics, ecology and evolution (Vasemägy and Primmer, 2005). However, despite enormous investment in human genomics, a large gap still exists in predicting the phenotype from the genotype (Makowsky et al., 2011). The need for understanding all steps in the decoding of the DNA message explains the interest in gene expression and its regulation, and therefore parallel progress to the genomic revolution has included transcriptome analysis (Ranz and Machado, 2006; Stapley et al., 2010; Ozsolak and Milos, 2011) and proteome analysis (Diz et al., 2012). However, gene expression does not end at the transcriptomic level, and a detailed understanding of the cellular work processes and responses requires the study of all steps during gene regulation and their final products at the proteome level (Diz et al., 2012). Proteomics complements functional genomics approaches, including microarray-based expression profiles (Shoemaker and Linsley, 2002), systematic phenotypic profiles at the cell and organism level (Gerlai, 2002), systematic genetic (Tong et al., 2001; Hannon, 2002) and small-molecule-based arrays (Kuruvilla et al., 2002). Ideally one may think that genome annotations should be completely proteomics driven. Indeed, large-scale proteomics data were early recognized as a potentially rich source for validation and reevaluation of genome annotations (Mann and Pandey, 2001).

II. JUSTIFICATION AND OBJECTIVES

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As mentioned in the introduction, the Manila clam *R. philippinarum* is a very important economic resource in many countries. The culture of Manila clam trends to raise year after year and the economic market and jobs generated by this industry, directly and indirectly, is very high in number. The carpet shell clam *R. decussatus* is also a very well appreciated species in the coastal European countries, where it is a native species, due to its taste and its ability to endure out of the water longer than other clam species, which allows better marketing. Infection with *Perkinsus olseni* is considered the main pathological threat for production of both clam species, causing mass mortalities in various locations of different countries, which justifies efforts to get knowledge on this clam disease looking for ways to minimise disease effects. *P. olseni* and *P. marinus* are included in the list of notifiable diseases of the World Organisation for Animal Health, but information on the former is scarce compared with that of the latter. On the parasite side, there is not information on genetic variability and occurrence of races of *P. olseni,* whereas the occurrence of races of *P. marinus* with different virulence has been demonstrated. Identification of races with different virulence is a key issue for zoo-sanitary prophylaxis; furthermore, knowledge on virulence factors that allow the parasite to proliferate through host tissues could help to design fighting ways against disease. On the host side, the biochemical bases of the Manila clam immune response against *P. olseni* and the factors determining if the infection progresses or is arrested are poorly understood.

Fighting against epidemic diseases affecting molluscs in the marine environment is very difficult when the etiological agent is well established in an area because, even if effective therapeutic agents were identified, their application into molluscs living in the open marine environment would be extremely complicated or useless (Villalba and Figueras, 2011). The lack of a true adaptive immune system in molluscs makes useless classical vaccination strategies; eradication of a well-established pathogen from a marine area is very complex, measures involving changes of culture procedures and fishery management can contribute to palliate disease effects to some extent but the most promising strategy is the use of disease-resistant (or tolerant) mollusc strains/species (reviewed by Villalba and Figueras, 2011). Production of disease-resistant mollusc strains through selective breeding programmes have been traditionally based on using long-term survivors living in disease affected areas as brood-stock; identification of resistance biomarkers to be used as criteria to select brood-stock should contribute to increase efficiency (higher success in shorter term) of the selective breeding programmes to produce disease-resistant strains (Villalba and Figueras, 2011).

The aim of this study was to gain knowledge (1) on the *P. olseni* variability through the Spanish coast, with emphasis on variability of virulence, and (2) on the modulation of the Manila clam protein profiling by infection with *P. olseni*, with emphasis on the search for protein markers of resistance to this infection. The

variability of *P. olseni* was addressed by analysing the genetic population structure as well as by proteomic comparison of *P. olseni* clones from locations scattered through the Spanish coast. Comparison of the proteome of *P. olseni* with those of two other *Perkinsus* spp., *P. marinus* and *P. chesapeaki*, was addressed to increase the variability scope. The analysis of modulation of host protein profile by infection with *P. olseni* was focused on haemocytes and plasma, aiming to concentrate attention on modulation of the clam immune response by the parasite; short and longer-term effects of the infection were considered.

The present dissertation has the following specific objectives:

1. Evaluation of the genetic variability of *P. olseni* through the Spanish coast using microsatellite markers.

2. Evaluation of the variability of the cellular and extracellular protein profiles of *P. olseni* through the Spanish coast.

3. Comparison of the cellular protein profiles of three *Perkinsus* spp.: *P. olseni*, *P. marinus* and *P. chesapeaki.*

4. Identification proteins of the haemocyte and plasma of the clam *R. philippinarum* of which expression is modulated by infection with *P. olseni*.

5. Identification of protein biomarkers of resistance to *P. olseni* infection in the clam *R. philippinarum.*

III. EVALUATION OF THE GENETIC VARIABILITY OF *Perkinsus olseni* AMONG REGIONS OF THE SPANISH COAST USING MICROSATELLITE MARKERS

The content of this chapter has been published in:

- Vilas, R., Cao, A., Pardo, B.G., Fernández, S., Villalba, A., Martínez, P., 2011. Very low microsatellite polymorphism and large heterozygote deficits suggest founder effects and cryptic structure in the parasite *Perkinsus olseni*. Infect. Gen. Evol. 11,904-911.

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III.1. ABSTRACT

Twelve microsatellite markers were used to characterize 130 clonal cultures of *Perkinsus olseni* derived from 30 clams from six different geographic locations. Only two loci were polymorphic in the four populations studied from Spanish coast (mean sample size = 31.2), and a third locus was variable in only two populations. In contrast, five parasites isolated from five clams from Japan and New Zealand showed variation at nine loci. Low genetic variation (2.08 ± 0.64 alleles per locus; mean genetic diversity: 0.101 ± 0.022), and very high F_{IS} values (0.857 on average) were observed in Spanish populations. A total of 39 multilocus genotypes (MLGs) were identified in the whole sample (121 clonal isolates after excluding incomplete MLGs due to missing data). A three-level hierarchical analysis of molecular variance found significant levels of genetic variation within infrapopulations (all the parasites in a single host; $Ø_{IS}$ = 0.679) and among infrapopulations within the component population (all the parasites among a host population; $Ø_{SC}$ = 0.579). Differences among the component population from different geographic locations were not significant ($Ø_{CT}$ = 0.057). These results suggest that an important fraction of F_{IS} is explained by the Wahlund effect, but also strong inbreeding within infrapopulations. Another explanation for the high F_{IS} within infrapopulations is the presence of haploid and diploid stages in the clam. Although fully aquatic system provides many opportunities for mixing of parasites from different clams, results are consistent with the consideration of all *P. olseni* in a clam as a cohesive genetic unit (i.e., deme). If the parasite was introduced into the Spanish coast with the importation of infected clams from Asia and Oceania, the low microsatellite polymorphism could be reflecting founder effects in the recent evolutionary history of *P. olseni*. The loss of alleles would be intensified in a scenario structured in numerous demes because of recurrent founder effects at microgeographic level.

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III.2. INTRODUCTION

The protozoan *Perkinsus olseni* is a parasite infecting a wide variety of bivalves around the world. It has been associated with extensive mortalities of commercially important species including oysters, clams, cockles and abalones (Villalba et al., 2004). The species was originally described in Australia (Lester and Davis, 1981) and is common in the Asian-Pacific region (Choi and Park, 2010). It has also been reported in Portugal (Azevedo, 1989), Spain (Robledo et al., 2000; Casas et al., 2002a), Italy (Abollo et al., 2006), France (Arzul et al., 2012), and Uruguay (Cremonte et al., 2005). Because of its significant impact on aquaculture, the infection by *P. olseni* is included in the OIE (Office International des Epizooties) list of diseases to be notified in order to prevent its spread. On the basis of its geographic distribution it has been hypothesized that the presence of the parasite in the Atlantic and the Mediterranean is due to imports of infected hosts from Asia and Oceania.

Despite substantial economic losses caused by *Perkinsus* species, little is known about relevant population parameters such as genetic variability, patterns of dispersal, population genetic structure, and reproductive strategies. However, this knowledge is essential for the efficient management of the parasitic disease (Tibayrenc and Ayala, 2002). The trophozoite of *P. olseni* occurs in clams as a single cell which undergoes vegetative multiplication. The trophozoite adopts a multi-nucleated form and gives rise to new cells. Once the host dies, trophozoites in the anaerobic condition provided by the necrotic tissue enlarge and transform into hypnospores, a resistant dormant stage with a thick external wall. When the hypnospores are in contact with aerated seawater at appropriate temperature and salinity, they undergo zoosporulation and thus the occurrence of new successive cell bipartitions within the original hypnospore wall and the generation and releasing of multiple cells of motile bi-flagellated zoospores capable of infecting new hosts (Villalba et al., 2004; Choi and Park, 2010). It is unknown if the life-cycle includes meiosis, fusion of gametes and zygote formation. Because clonal amplification occurs both within and outside the host it is expected that clonal propagation shapes the population genetic structure. Therefore, populations of *P. olseni* may be characterized by different manifestations of clonality, such as linkage disequilibrium, fixed heterozygosity within loci, deviation from Hardy–Weinberg expectations towards a heterozygote excess, and the presence of overrepresented identical multilocus genotypes (Tibayrenc and Ayala, 2002; De Meeûs et al., 2006).

The inference of clonal structure in organisms that may alternate clonal amplification and sexuality requires the definition of what constitutes a cohesive genetic unit (i.e., deme), because cryptic genetic structuring within the units defined as demes can both hide the expected excess of heterozygotes under clonality and overestimate the rate of sexual recombination (Halkett et al., 2005; Prugnolle and De Meeûs, 2010). For parasitic organisms, the deme could be represented by individual

infrapopulations or the component population. The infrapopulation refers to all individuals of a parasite species in an individual host at a particular time and the component population refers to all infrapopulations at a given place and time (Bush et al., 1997). It is even possible that other different units constitute the deme, such as groups of infrapopulations (e.g., family groups of hosts, different host species…) and temporal groups distributed over several infrapopulations. Thus, the entity we call deme, an evolutionary unit characterized by certain persistence over time, may be very different depending on the life cycle of the parasite and the ecology of transmission (Criscione et al., 2005; Criscione and Blouin, 2006).

Microsatellites are useful markers to investigate patterns of population genetic structure of parasitic protozoa. For instance, analyses of microsatellite variation revealed evidences of clonal structure in *Sarcocystis* (Asmundsson et al., 2006), *Toxoplasma* (Ajzenberg et al., 2004), *Plasmodium* (Razakandrainibe et al., 2005), and *Trypanosoma* (Simo et al., 2010). Microsatellite studies also revealed that *Trypanosoma, Leishmania, Toxoplasma, Cryptosporidium*, and *Plasmodium*, whose mode of reproduction has often been assumed to be predominantly clonal, actually display different population structures, ranging from clonality to panmixia (Anderson et al., 2000; Ajzenberg et al., 2004; Morrison et al., 2008; Koffi et al., 2009; Rougeron et al., 2009). Here, we carry out a study of genetic variation of *P. olseni* from the three main areas of clam production in Spain. We found very low microsatellite polymorphism, particularly in comparison with a sample from the Pacific area, which suggests strong genetic drift probably caused by recurrent founder effects. We also observed strong heterozygote deficits in the component population from different locations, indicating subdivision at some level below and suggesting that the infrapopulation constitutes the deme in *P. olseni*.

III.3. MATERIALS AND METHODS

III.3.1. Parasite isolates and microsatellite markers

A total of 130 clonal cultures of *P. olseni* from six geographic regions were genotyped for 12 microsatellites. Five clonal cultures per individual host were analyzed from four locations through the Spanish coast: Ría de Arousa and Ría de Pontevedra in Galicia (NWSpain), Carreras River in Huelva (Andalucía, SW Spain), and Delta de l'Ebre in Catalonia (NE Spain) (Fig. III.1). Therefore, the three main areas of clam production in Spain (Galicia, western Andalucía and Catalonia) were included in the study. Five carpet shell clams *(Ruditapes decussatus*) from Arousa, four from Pontevedra, eight from Huelva, and eight Manila clams (*Ruditapes philippinarum*) from Catalonia were used to isolate *P. olseni*. The parasites were isolated from the gills and were allowed to proliferate *in vitro* as described by Casas et al. (2002b). The in vitro cultures (one per

host) were cloned by limiting dilution plating in 96-well culture plates, and five monoclonal derivatives of each isolate culture were expanded. Additionally, three *P. olseni* clonal cultures from three Manila clams collected in Japan and two more clonal cultures from two clams *Austrovenus stutchburyi* taken in New Zealand were used in the analyses. Parasites were previously identified as *P. olseni* by using a PCR-RFLP diagnostic assay of the rRNA internal transcribed spacer (ITS) region (Abollo et al., 2006). We studied the 12 microsatellite loci (PolUSC1-PolUSC12) previously described in *P. olseni* following the protocols for DNA extraction and microsatellite genotyping reported (Pardo et al., 2011). These markers confirmed that all *Perkinsus* isolates belong to a single species. The presence of *Perkinsus mediterraneus*, the other species reported from the Spanish coast (Casas et al., 2004), in the studied samples may be discarded because several markers suitable for distinguishing the two species were used (Pardo et al., 2011).

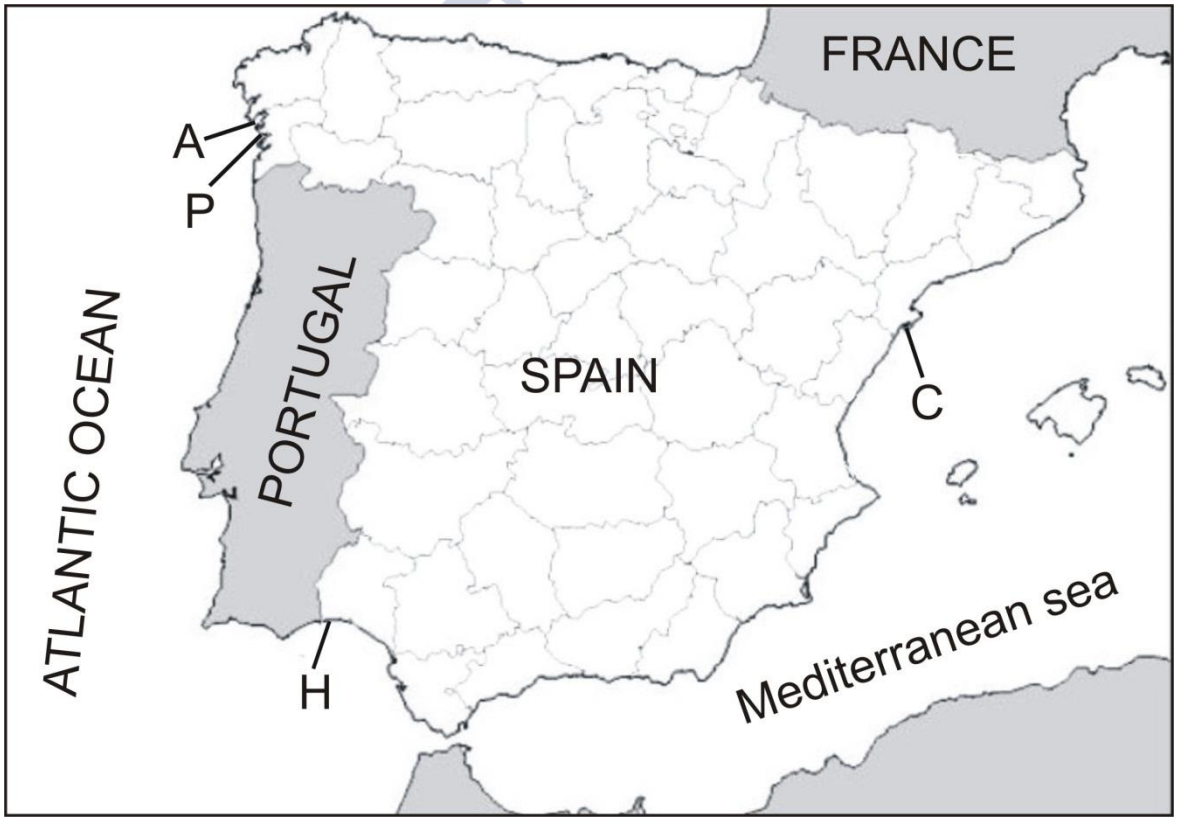

Fig. III.1. Map showing the four Spanish locations where infected clams were collected: Ría de Arousa (A) and Ría de Pontevedra (P) in Galicia, Carreras River in Huelva, Andalucía (H) and Delta de l'Ebre in Catalonia (C).

III.3.2. Data analysis

Allele frequencies and estimates of genetic variation within populations (average number of alleles per locus, allelic richness, and heterozygosity) were calculated using FSTAT 2.9.3.2 (Goudet, 2001). After excluding clonal cultures with missing data (the number of isolates decreased from 130 to 121), genotypic diversity

(G) was measured as the number of different multilocus genotypes (MLGs) over the total number of clonal cultures per geographic sample (n). All clonal cultures from one clam from Huelva showed incomplete MLGs due to missing data, so they were not used in the MLG analysis. Conformity to Hardy–Weinberg proportions was tested using exact test as implemented in GENEPOP 3.4 (Raymond and Rousset, 1995). Linkage disequilibrium between pairs of loci was tested with the randomization test based on the log-likelihood ratio G of genotypic frequencies from paired loci in contingency tables. Genotypes at two loci are associated at random a number of times and the log-likelihood G test statistic is recalculated on the randomized data set. However, tests were not possible in many cases due to the low microsatellite variation of *P. olseni*. We adjusted the P-values with the sequential Bonferroni correction. Linkage disequilibrium analyses were performed in FSTAT. Population structure was inferred by using the Weir and Cockerham's (1984) unbiased estimators of Wright *F*-statistics (Wright, 1965) defined for three hierarchical levels (individuals within geographical populations, geographical populations within metapopulation, and metapopulation): F_{IT} measures an overall inbreeding combining the homozygosity of individuals within populations relative to that measured between individuals (F_{IS}) and the homozygosity caused by the Wahlund effect when several differentiated populations are treated as a single panmictic unit (F_{ST}). These statistics can be translated into biologically characteristics relevant to molecular epidemiology (De Meeûs et al., 2007). For instance, the three *F*-statistics equals zero in a single panmictic population. Although the sample size per infrapopulation is small (only a maximum of five clonal cultures of *P. olseni* per clam), we calculated the mean F_{IS} among infrapopulations because the number of polymorphic infrapopulations is relatively high. *F*-statistics were calculated with FSTAT and their significant deviation from zero was tested by randomizing alleles between individuals within subsamples for F_{IS} and randomizing individuals among subsamples for F_{ST}. Randomizations (10,000) were done in FSTAT. The hierarchical distribution of genetic variation was also characterized using analysis of molecular variance (AMOVA). Three-level AMOVA was conducted in ARLEQUIN 3.0 (Excoffier et al., 2005) using an F_{ST}-like estimator under the infinite alleles model (IAM; Weir and Cockerham, 1984) and an R_{ST}-like estimator under the stepwise mutation model (SMM; Michalakis and Excoffier, 1996), since results may vary according to the assumptions of different mutation models (Balloux and Goudet, 2002; Balloux and Lugon-Moulin, 2002). The analysis was carried out to assess the amount of variance imputable to genetic differences between geographic populations ($Ø_{CT}$), among infrapopulations within geographic populations ($Ø_{SC}$), among individuals within infrapopulations ($Ø_{IS}$), and within individuals ($Ø_{IT}$). One thousand permutations were generated to assess whether levels of differentiation were significantly greater than zero (Excoffier et al., 2005). Genetic differentiation between geographical samples was also estimated with D_c genetic distance (Cavalli- Sforza and Edwards, 1967), calculated using POPULATIONS 1.2.26 (Langella, 2002). A Neighbor-joining (NJ) tree of individuals (clonal isolates)

derived from shared allelic distance (Chakraborty and Jin, 1993) was built with the program POPULATIONS. The tree was visualized using TREEVIEW 1.4 (Page, 1996).

III.4. RESULTS

III.4.1. Genetic variability

Low genetic variation was detected in *P. olseni* samples from the Spanish coast. The average number of alleles per locus was 2.08 ± 0.645, ranging from one to eight, and the mean genetic diversity in Spanish populations was H_s = 0.101 ± 0.022 (Table III.1).The number of polymorphic loci within populations (0.95 criterion) ranged from two (Pontevedra and Huelva) to three (Arousa and Catalonia), and the expected heterozygosity of polymorphic loci ranged from 0.025 to 0.711 (Table III.2). Samples from the Pacific Ocean (three clonal isolates from Japan and two from New Zealand) showed variation at nine loci. When the five parasites from the Asian-Pacific region were included in the analysis the average number of alleles per locus increased until 3.25 ± 0.733. This increase indicates high genetic variation and differentiation of these parasites with respect to those from the Spanish coast. After excluding individuals with missing data, a total of 39 distinct MLGs were identified in the whole data set (121 clonal cultures). Genotypic diversity within populations was low in Pontevedra and Huelva and moderate in Arousa and Catalonia (TableIII.1). The five MLGs from Japan and New Zealand were unique.

Table III.1. Genetic variability in geographic populations of *P. olseni*. Sample size (*N*), sample size after excluding incomplete multilocus genotypes because missing data (*n*), Nei's unbiased genetic diversity (H_s), average number of alleles (*A*), mean allelic richness per locus and population (*AR*), number of different complete multilocus genotypes (MLGs) and genotypic diversity (*G*). The number of individual hosts is shown in parentheses.

	N	H_s	*A*	*AR*	*n*	MLGs	*G*
Arousa	25 (5)	0.128	1.58	1.56	25 (5)	13	0.52
Pontevedra	20 (4)	0.062	1.42	1.42	20 (4)	7	0.28
Huelva	40 (8)	0.067	1.42	1.36	32 (7)	7	0.22
Catalonia	40 (8)	0.149	1.83	1.69	39 (8)	16	0.41
Japan	3 (3)	-	1.50	-	3 (3)	3	1.0
N. Zealand	2 (2)	-	1.44	-	2 (2)	2	1.0

Table III.2. Genetic diversity at three polymorphic loci of 130 clonal isolates of *P. olseni* from Spain. Number of alleles (*A*), the total number of alleles per locus is shown in parentheses; expected heterozygosity (*H*) and deviation of the observed genotypic frequencies from the Hardy-Weinberg expectations (F_{IS}). All F_{IS} values except for PolUSC8 in Pontevedra are statistically significant ($P < 0.001$)

Locus	Arousa	Pontevedra	Huelva	Catalonia	Mean ± SE
PolUSC7					
A (8)	3	5	4	6	
H	0.530	0.555	0.523	0.711	0.580 ± 0.044
F_{IS}	1.000	0.820	0.940	0.856	0.904 ± 0.040
PolUSC8					
A (5)	4	2	2	4	
H	0.585	0.145	0.262	0.594	0.396 ± 0.114
F_{IS}	0.863	0.655	1.000	0.832	0.837 ± 0.071
PolUSC4					
A (3)	3	1	2	3	
H	0.418	0.000	0.025	0.479	0.230 ± 0.109
F_{IS}	0.801	-	-	0.945	0.8730.072

III.4.2. HWE deviations and genotypic disequilibrium

There was a large deficit of heterozygotes within populations compared with genotypic frequencies predicted from Hardy–Weinberg expectations (Table III.2). The population F_{IS} for all loci were: 0.893 (Arousa), 0.786 (Pontevedra), 0.931 (Huelva), and 0.872 (Catalonia). The overall mean value was 0.857 ± 0.043 (Table III.2). All F_{IS} values except PolUSC8 in Pontevedra (P = 0.085) differed from the null hypothesis (FIS = 0; P < 0.001). The mean F_{IS} value within infrapopulations was 0.502 ± 0.087. Given the low variation detected in the four Spanish populations, testing random association of genotypes at different loci was only possible for 10 pairs of loci. None of these pairs show significant linkage disequilibrium.

III.4.3. Distribution of MLGs within and among hosts

Only one MLG (10) was found in all four Spanish populations reaching a frequency of 0.269 in the whole Spanish sample. The remaining MLGs did not exceed the 0.1 frequency (Table III.3). In 79.2% (19 out of 24) of clams from Spain, more than

one parasite MLG was found among the four or five genotyped, and in 29.2% of them, three or more MLGs were found. These figures are high considering the low number of clonal isolates per clam (mean = 4.65) and the low genetic variability detected. The 66.7% (16 out of 24) of clams from Spain harbored at least one MLG not present in any other clam. The corresponding values within populations ranged from 28.6% (two out of seven) in Huelva to 100% (five out of five) in Arousa. The five clams from the Pacific area harbored unique MLGs. The 82% MLGs (32 out of 39) appeared in a single clam and the most frequent MLG was only present in 27.6% of clams (Table III.3). These results show a high level of genetic structure among infrapopulations.

III.4.4. Population structure and differenciation

Genetic structure among geographic populations from Spain was moderate but significant (F_{ST} = 0.137, P < 0.001). The large difference between the F_{ST} and the overall measure of deviation from panmixia (F_{IT} = 0.897) is consistent with high levels of inbreeding (F_{IS} = 0.881). All pairwise F_{ST} values except the one between Pontevedra and Huelva were significant (P < 0.008; Table III.4). Absolute genetic distances between geographical populations closely reflect F_{ST} values (Table III.4). No correlation was detected between genetic and geographical distances. In fact, the closest geographical populations (Pontevedra and Arousa) were genetically more related to Huelva and Catalonia, respectively (Fig. III.1; Table III.4).

These results do not support isolation by distance model in Spanish coast. The Neighbor-joining tree based on shared allelic distances between MLGs shows that (1) the parasites from the Pacific area are highly differentiated, particularly those from New Zealand; and (2) differentiation between individuals from Spain is not associated with their geographic origin (Fig. III.2). The three-level hierarchical AMOVA showed significant levels of genetic differentiation between infrapopulations within geographic populations, between individuals within infrapopulations, and between individuals under IAM and SMM assumptions (Table III.5). After removal the variation attributable to different hierarchical levels including differences among individuals within infrapopulations, differentiation among geographic populations was not significant. The proportion of genetic variation was distributed similarly at all levels under both mutation models (although it was distributed more evenly across levels under the SMM). The largest proportion of variation was explained by differences among infrapopulations within geographic populations and to a minor extent between individuals within infrapopulations. Fixation indices confirmed that greater variation was observed among infrapopulations rather than between geographical populations (Table III.5).

Table III.3. Abundance and distribution of the 39 MLGs detected in clams from Arousa (Aa-Ae), Pontevedra (Pa-Pd), Huelva (Ha-Hi), Catalonia (Ca-Ch), Japan (JAPa-JAPc) and New Zealand (NZa and NZb). Individuals with missing data were excluded.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Aa	4	1																	
Ab			3	1	1														
Ac						1	1	2	1										
Ad										3	2								
Ae	3											1	1						
Pa														1	3	1			
Pb										4							1		
Pc	1									4									
Pd										3								1	1
Ha															4				
Hc																			
Hd			5																
He														1	4				
Hf										5									
Hg										5									
Hh										5									
Ca							4												
Cb																			
Cc										2				3					
Cd														5					
Ce							4												
Cf																			
Cg																			
Ch																			
JAPa																			
JAPb																			
JAPc																			
NZa																			
NZb																			

	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
Aa																				
Ab																				
Ac																				
Ad																				
Ae																				
Pa																				
Pb																				
Pc																				
Pd																				
Ha	1																			
Hc		1	1																	
Hd																				
He																				
Hf																				
Hg																				
Hh																				
Ca				1																
Cb					4	1														
Cc																				
Cd																				
Ce							1													
Cf								1	2	1	1									
Cg							2					3								
Ch													1	2	1					
JAPa																1				
JAPb																	1			
JAPc																		1		
NZa																			1	
NZb																				1

Table III.4. Pairwise F_{ST} values among populations (below diagonal) and Cavalli-Sforza and Edwards D_c distances (above diagonal). All F_{ST} values except that between Huelva and Pontevedra are significant (P < 0.008).

	Arousa	Pontevedra	Huelva	Catalonia
Arousa	-	0.100	0.091	0.087
Pontevedra	0.136	-	0.052	0.100
Huelva	0.135	0.000	-	0.082
Catalonia	0.094	0.179	0.174	-

Table III.5. Three-level hierarchical AMOVA (population, infrapopulation, individual) for *P. olseni*. Estimates were obtained under the assumptions of an infinite alleles model (IAM) and stepwise mutation model (SMM). Permutation tests for deviation from zero differentiation were highly significant (P<0.001) at all levels except among geographic populations (P = 0.153 and 0.222 under the IAM and SMM, respectively).

		Percentage of variation		Fixation indices	
	d.f.	IAM	SMM	IAM	SMM
Among geographic populations	3	5.70	3.89	$Ø_{CT}$ 0.057	0.039
Among infrapopulations within geographic populations	21	54.60	48.73	$Ø_{SC}$ 0.579	0.507
Among individuals within infrapopulations	99	26.97	33.98	$Ø_{IS}$ 0.679	0.717
Within individuals	124	12.73	13.40	$Ø_{IT}$ 0.873	0.866

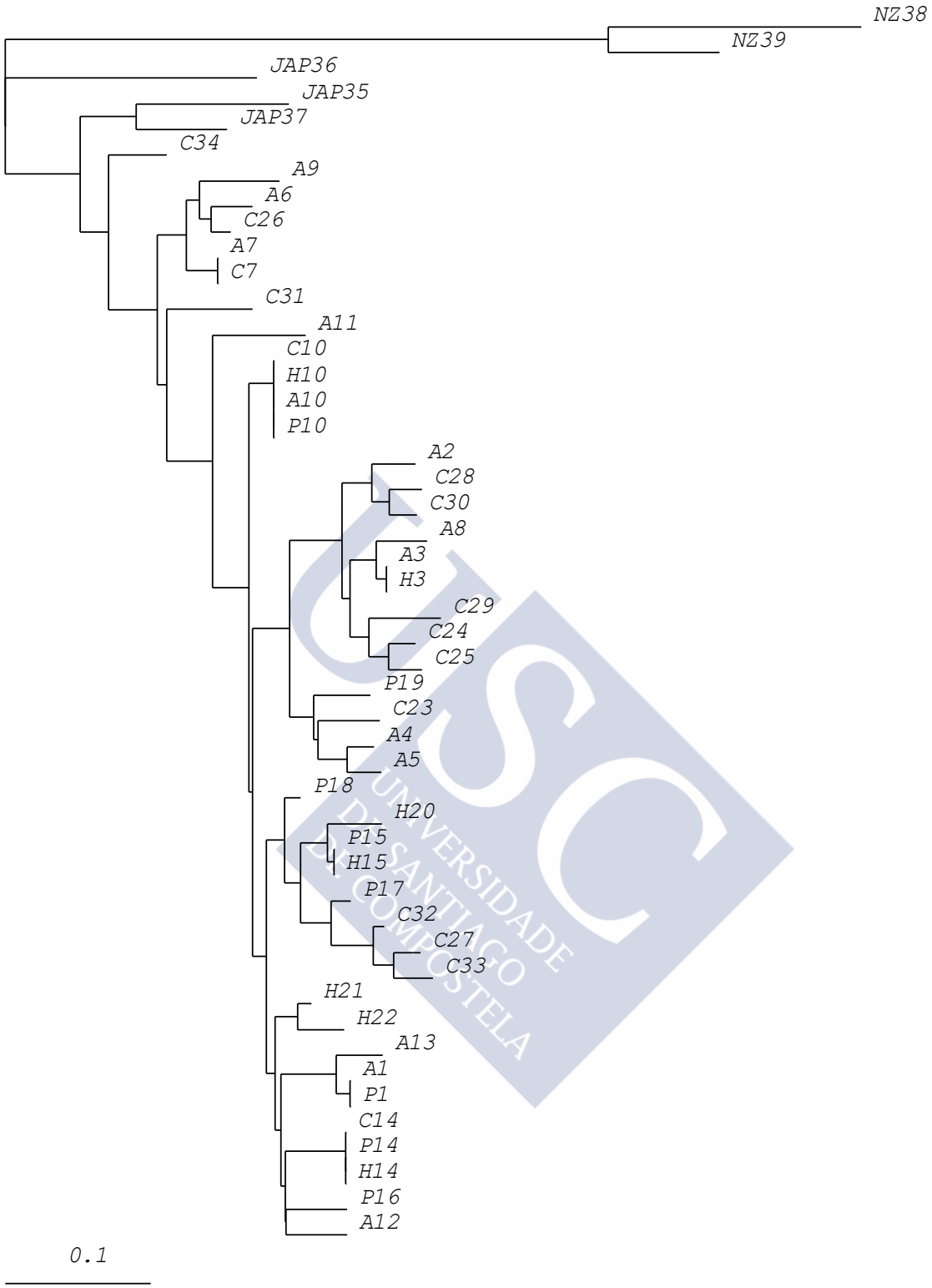

Fig. III.2. Neighbor-joining tree based on shared allelic distances between clonal isolates. Only one representative of each MLG (numbers) was used within geographical populations (letters as in Fig. III.1). The tree, which includes samples from Japan (JAP), was rooted with the samples from New Zealand (NZ).

III.5. DISCUSSION

The analysis of variation at 12 microsatellite loci of *P. olseni* from different geographical locations revealed strong heterozygote deficits relative to Hardy–Weinberg expectations, low polymorphism and number of alleles, moderate genetic structure among geographical populations and high genetic structure among parasites from different clams. It is also notable the frequent finding of several genetically different clonal isolates obtained from a single individual host and the high differentiation of samples from the Asian-Pacific area in comparison with those from the Spanish coast. Despite the observed genotypic deficits, the presence of heterozygotes shows that the parasite has a diploid phase of the life cycle and confirms sexual reproduction, an observation in accordance with previous studies in *Perkinsus marinus* (Reece et al., 2001; Thompson et al., 2011). Assuming that all parasites analyzed are diploid, as suggested by the observation of a single cell type isolated for culture and the presence of heterozygotes, the strong deficiency of heterozygotes detected within geographical samples of *P. olseni* is inconsistent with a strictly clonal reproduction model. Theoretical studies have shown that diploids with predominant clonality exhibit negative F_{IS} values (Balloux et al., 2003; De Meeûs and Balloux, 2004). However, a parasitic species may be highly clonal and still show high FIS values if the sample analyzed as a single population actually represents several demes (Prugnolle and De Meeûs, 2010). The Wahlund effect explains a significant part of the observed heterozygote deficiencies within geographical samples because infrapopulations largely differ in their MLG composition, as shown by hierarchical analysis of the distribution of the genetic variation. Genetic distances among clonal isolates from different clams collected at four Spanish locations are relatively high regardless the geographic origin and infrapopulations showed significant genotypic differentiation. Therefore, the treatment of several infrapopulations from the same locality as a single panmictic unit would result in positive F_{IS} values. This suggests that the component population of *P. olseni* is subdivided in genetically differentiated demes, probably represented by individual infrapopulations. If the component population is structured in numerous demes, the parasite may show clonal structure despite the high F_{IS} values observed within geographical populations. However, we found strong heterozygote deficits within infrapopulations. Although the sample size per infrapopulation is not large, 19 polymorphic infrapopulations showed a very high mean F_{IS} value, which suggests inbreeding if the infrapopulation is not structured (i.e. it is the deme). This result was confirmed by the AMOVA analysis. It is known that the parasite undergoes vegetative multiplication within the host as well as in sea water (Villalba et al., 2004), so inbreeding resulting from crosses between genetically identical parasites might be common. Extreme inbreeding in part caused by frequent recombination between genetically identical cells was recently observed in other parasitic protozoan that alternates clonality and sexuality in its life cycle (Rougeron et

al., 2009). Positive F_{IS} within infrapopulations of *P. olseni* suggest frequent sexual recombination and no clonal population structure. In addition, taking into account the low number of clonal cultures analyzed per individual host and the low number of alleles detected, the fraction of clams infected by parasites with different MLGs is large (79.2%), a result that does not fit well with a highly clonal organism. The analysis of hierarchical components of genetic variation showed large differences among individuals within infrapopulations and among infrapopulations within the component population from four geographic locations. Since a large proportion of the genetic variation was observed between infrapopulations, the Wahlund effect is an important cause of the observed heterozygote deficits at the component population level. Therefore, results suggest both the Wahlund effect due to the subdivision of the component population in numerous demes and strong heterozygote deficits within infrapopulations (which may represent demes), probably caused by frequent mating between individuals belonging to the same clone. However, in order to appropriately evaluate the hypothesis of inbreeding and the Wahlund effect it would be necessary to increase the sample size to properly estimate allele frequencies within infrapopulations. Other explanations for the genotypic deficits detected seem less likely. Although microsatellite null alleles are commonly encountered in population studies (Dakin and Avise, 2004), it is unlikely that they explain the observed heterozygote deficiencies because F_{IS} values per locus were very high at all polymorphic loci and they showed little variation across loci in all populations. The selection hypothesis is also unlikely because all polymorphic markers are independent anonymous non-coding loci and it is expected that the heterozygous disadvantage is infrequent in nature due to its instability (Hedrick, 2009). Understanding the infrapopulation as a deme means that *P. olseni* from the same clam defines a cohesive group through genealogical relationships. The infrapopulation should undergo recurrent generations and persist over time, thus showing random variation in allele frequencies from one generation to the next. This is possible if offspring of the same infrapopulation tends to infect again and again their natal host or if it is transmitted together from host to host over several generations (Criscione et al., 2005). Although the parasite life cycle does not appear to involve intermediate hosts, which could contribute to the spread of the parasite, and clams exhibit low mobility, this scenario is unexpected in a fully aquatic system. Another possibility is that *P. olseni* can complete its life cycle within the host. Under these conditions of low mixing of parasites from different individual host, it is expected low genetic variation within infrapopulations and high genetic differentiation among them due to genetic drift. Largely homozygous populations with low number of alleles are coherent with a model characterized by population fluctuations and chance colonization events (Criscione et al., 2005). Thus, it is likely that each clam is infected by a few cells which proliferate rapidly in the host tissue, so that differences among infrapopulations could be the result of a founder-like effect. Because we are assuming that all individuals analyzed are diploid and the ploidy

of this species has not been experimentally confirmed, caution should be taken before rejecting the clonal structure hypothesis. If many of the 91 clonal isolates (the 75.2% of the total sample after excluding incomplete MLGs) whose genotypes were interpreted as homozygous at all loci were haploid, the F_{IS} would be inflated within infrapopulations, thus erasing the excess of heterozygotes expected under predominant clonal reproduction. Under the assumption of diploidy the increase of F_{IS} caused by the presence of haploid forms would be interpreted as result of inbreeding. Therefore, *P. olseni* may still have clonal structure if two different life stages, one haploid and one diploid, are present in clam infection (something likely if the parasite was able to complete its life cycle within the host) and all individuals analyzed did have not the same ploidy. This consideration is relevant because phylogenetic analyses place *Perkinsus* basal to dinoflagellate lineages in which several taxa are known to be haploid with ephemeral diploid stages (Thompson et al., 2011).

We only analyze two and three clonal cultures from two and three clams collected at New Zealand and Japan, respectively. However, both samples revealed a mean number of alleles similar to that estimated in all Spanish populations where a mean sample size of 31.2 was collected. Because the number of alleles is very sensitive to the sample size, this result suggests that populations from Japan and New Zealand are much more variable than those from Spain. This is consistent with the hypothesis that the parasite has been introduced from the Asian-Pacific area through the importation of infected clams into Spain. The concomitant founder effect would explain a severe loss of variation in Spanish populations, particularly in the number of alleles. This hypothesis is also coherent with the moderate population genetic structure observed in the Spanish coast. Thus, higher variation was observed among infrapopulations rather than among geographical populations suggesting high genetic sub-structuring related with transmission dynamics. Furthermore, the lack of correlation between genetic and spatial distances suggests that the dispersal of the parasite favored by ocean currents is a factor that weighs less than an external colonization due to imports of infected clams in shaping the population genetic structure of the parasite. Extremely low microsatellite polymorphism probably associated to population bottlenecks was also observed in other parasitic protozoan (Leclerq et al., 2004). Although strictly aquatic system provides multiple opportunities for the mixing of *P. olseni* from different clams, results suggest that the infrapopulation serves as a deme. The low number of alleles detected compared with a sample from the Pacific area is consistent with a founder effect associated with the importation of infected clams. The loss of overall genetic diversity caused by this phenomenon would be intensified if the infrapopulation constitutes the deme because of recurrent founder effects at microgeographic level and substantial decreasing of the component population effective size due to subdivision. A combination of factors such as the occupation of small discontinuous environments represented by individual clams, recurrent founder effects and large deme fluctuations probably lead to strong genetic

drift thus explaining the low number of alleles observed. Further studies aimed to identifying ploidy levels of *P. olseni* within clams and additional population genetic structure analysis involving larger sample sizes within infrapopulations are needed to estimate the rate of clonal reproduction and to determine whether cryptic structure is the main cause of the high homozygosity observed.

IV. VARIABILITY OF THE CELL PROTEOME OF *Perkinsus olseni* AMONG REGIONS OF THE SPANISH COAST

- **The content of this chapter has been accepted for publication in**:

Fernandez-Boo, S., Villalba, A., Cao, A. *In press*. Variability of the cell proteome of *Perkinsus olseni*, protistan parasite of molluscs, among regions of the Spanish coast. Dis. Aquat. Organ.

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IV.1. ABSTRACT

The variability of the proteome of *in vitro* cultured *Perkinsus olseni* cells deriving from 4 regions of the Spanish coast was evaluated. The regions involved were: the Rías of Arousa and Pontevedra (Galicia, NW Spain), Carreras River in Huelva (Andalusia, SW Spain) and Delta de l'Ebre (Catalonia, NE Spain). *P. olseni in vitro* clonal cultures were produced starting from parasite isolates from four individual clams from each region. Those clonal cultures were used to extract cell proteins, which were separated by 2D electrophoresis. Qualitative comparison of *P. olseni* protein expression profiles among regions was performed with PD Quest software. Around 700 protein spots from parasites derived from each region were considered, from which 141 spots were shared by all the regions. Various spots were found to be exclusive of each region. Higher similarity was found among the proteome of *P. olseni* from the Atlantic regions than between the Mediterranean location and the Atlantic ones. A total of 54 spots were excised from the gels and sequenced. Nineteen proteins were annotated after searching in databases, 13 being shared by all the regions and 6 exclusive of one region. Most identified proteins were clustered into glycolysis, oxidation/reduction process, metabolism and response to stress. From proteins set analysed, no direct evidence of *P. olseni* variability associated with virulence was found although the differences in metabolic adaptation and stress response could be connected to pathogenicity.

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IV.2. INTRODUCTION

The genus *Perkinsus* includes protistan parasites infecting a wide range of molluscs over the world (Villalba et al., 2011). The species *Perkinsus olseni* was first described infecting abalones *Haliotis ruber* in Australia (Lester & Davis, 1981). Since then, *P. olseni* has been reported from gastropod and bivalve molluscs of Australasia, Europe, Asia and South America (Villalba et al., 2011). The name *Perkinsus atlanticus*, given to a parasite of carpet-shell clams *Ruditapes decussatus* from Portugal (Azevedo 1989), was considered synonym of *P. olseni* (Murrell et al., 2002). Likely, *P. olseni* was introduced into Europe by commercial trade of Manila clams *Ruditapes philippinarum* from Asia coasts (Hine, 2001, Elandaloussi et al., 2009a); it has spread through Italy (Da Ros and Canzonier, 1985, Abollo et al., 2006), Portugal (Azevedo, 1989), Spain (Casas et al., 2002a, Elandaloussi et al., 2009b) and France (Goggin, 1992; Arzul et al., 2012). Most studies on the genus *Perkinsus* have been devoted to *P. marinus*, which has been causing mass mortalities in Eastern oyster *Crassostrea virginica* populations of the Atlantic and Gulf coasts of the USA for more than 50 years (Ray, 1996; Andrews, 1996; Burreson and Ragone Calvo, 1996). The World Organisation for Animal Health (OIE) has included *P. marinus* and *P. olseni* in the list of notifiable diseases (http://www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2014/).

Variability in virulence and other physiological aspects through populations of *P. marinus* has been demonstrated (La Peyre et al., 1995a; Bushek and Allen, 1996; Chu and Lund, 2006), which is consistent with the occurrence of genetic variability among geographic strains (Reece et al., 1997; Kotob et al., 1999; Reece et al., 2001; Thompson et al., 2011). The existence of races that vary in virulence or environmental tolerance has important management implications and spreading virulent races should be avoided. Spreading races with varying environmental tolerances can also be harmful by producing epidemic outbreaks in areas that are inhospitable to indigenous parasitic races (Bushek and Allen, 1996). Analysis of protein profiles is another way to search for variability and could be valuable for understanding biological processes including development, evolution and pathogenicity of these organisms. Proteomics involves the systematic analysis of gene expression at a protein level (Sperling, 2001; Lee et al., 2005). The proteomic techniques are powerful tools to compare different isolates of the same species (Shin et al., 2005; Regidor-Cerrillo et al., 2012) morphospecies (Chan et al., 2005), genetic variability (Mosquera et al., 2003), and differences in virulence among isolates or strains of the same species (Regidor-Cerrillo et al., 2012).

A proteomic approach using two-dimensional electrophoresis (2-DE) coupled to Mass Spectrometry (MS) was performed to compare the protein expression profiles between *P. olseni* clonal cultures deriving from four regions of the Spanish coast, as a

way to search for variability, which could be the basis of geographical differences in virulence and environmental adaptation of *P. olseni*.

IV.3. MATERIALS AND METHODS

IV.3.1. Production of in vitro *clonal cultures.*

Parasites *Perkinsus olseni* were isolated from 4 carpet shell clams *R. decussatus* from Ría de Arousa, 4 from Ría de Pontevedra in Galicia (NW Spain), 4 from Carreras River in Huelva (Andalucía, SW Spain) and from 4 Manila clams *R. philippinarum* from Delta de l´Ebre in Catalonia (NE Spain) (Fig.IV.1). The parasites were isolated from the gills and were allowed to proliferate *in vitro* as described by Casas et al. (2002b). The *in vitro* cultures (one per host) were cloned by limited dilution plating in 96-well culture plates (Casas and La Peyre, 2009). One monoclonal derivate of each isolate was *in vitro* expanded and used in the study. After 2 months, when the cultures were in exponential growth phase, parasites of each clonal culture were harvested; the viable cells were counted by staining with 50 mg/L neutral red and counting in a Malassez chamber. After centrifugation (800 x g, 10 min, 25 ºC), 5 x 10^6 cells were reseeded and the remaining cells were stored at -80 ºC for proteomic analysis. This process was repeated 5 more times up to collecting 150 x 10^6 cells of each clone. Species identification of the cells in each clonal culture was performed by PCR followed by restriction fragment length polymorphism assay as described by Abollo et al. (2006), thus confirming that the cells of every clon corresponded to *P. olseni*.

IV.3.2. Protein extraction.

A total of 150 x 10^6 frozen cells from each clonal culture were resuspended in 1 mL of lysis buffer (8M urea, 2M thiourea, 2% CHAPS, 1% DTT, 0.8% ampholites pH 3-10) during 2h 30 min for protein extraction. The protein concentration was determined by Lowry assay using the RC/DC Protein Assay Bio-Rad and measuring in a microplate lecture Expert 96 (Asys Hitech). Then 400 µg of protein were purified using the 2D CleanUp kit Bio-Rad and resuspended in 1 mL of rehydration solution (7M urea, 2M thiourea, 4% chaps, 0.3% DTT, 0.5% IPG buffer and bromophenol blue traces).

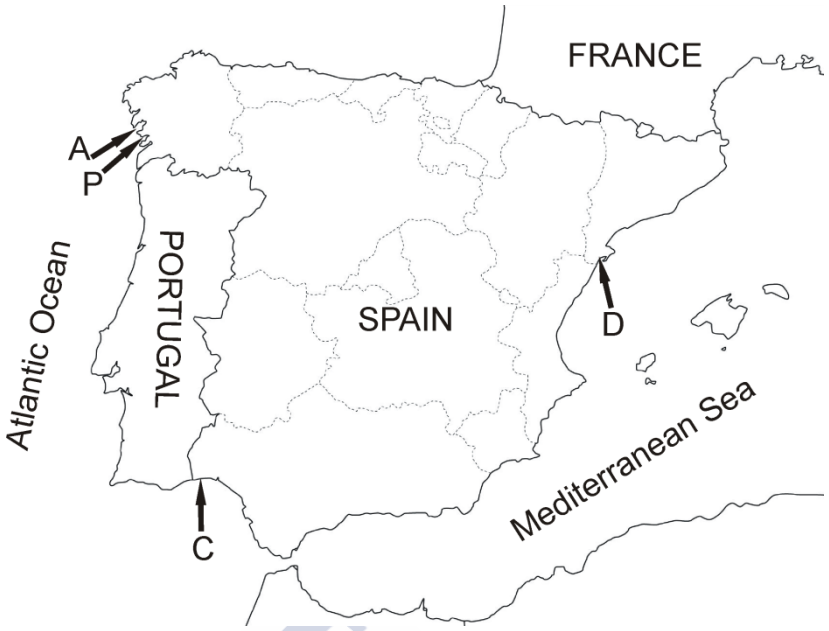

Fig. IV.1. Map showing the four Spanish locations where infected clams were collected: Ría de Arousa (A) and Ría de Pontevedra (P) in Galicia, Carreras River (C) in Huelva (Andalusia) and Delta de l'Ebre (D) in Catalonia.

IV.3.3. Two dimensional electrophoresis (2DE) and image analysis.

Preliminary trials had been performed to tune up the experimental conditions for separation of *P. olseni* proteins by 2DE. Each sample was processed in quadruplicate. For the first dimension, aliquots of 100 µg of protein were diluted to a final volume of 250 µL in rehydration solution. Samples were centrifuged for 1 min at maximum speed to remove bubbles and loaded onto the immobilised pH gradient (IPG)-strips (11cm, pH 5-8, Bio-Rad) by in-gel rehydration. Iso-Electro Focusing (IEF) was performed using a Protean®IEF System (Bio-Rad) at 20ºC, as follows: 50 V were applied during the rehydration step for 12 h, then the IEF proceeded through 5 steps, 150 V for 30 min, 300 V for 30 min, 1000 V for 60 min, 8000 V for 180 min and 8000 V until 35000 Vh. After IEF, the strips were incubated for 20 min in equilibration buffer (50 mM Tris–HCl pH 8.8, 6M urea, 30% glycerol, 2% SDS) with 10 mg/ml DTT to reduce the proteins and, in a second step, incubated for 20 min in this mix with iodoacetamide at 45 mg/ml to alkylate proteins. The second dimension involved separation in SDS-PAGE, which was carried out at 15ºC through 12,5% polyacrylamide homemade gels, using a Protean®II xi cell (Bio-Rad) in two steps: 15 mA/gel for 15 min and 50 mA/gel until separation was finished (≈3:30 h). Molecular markers from 250 kDa to 10 kDa (BioRad) were run in the second dimension next to the sample for protein size determination. Four gels were produced from each clonal culture. The gels were silver stained with a protocol compatible with MS analysis: (1) incubation in 50% (v/v) methanol 5% (v/v) acetic acid for 1 hour or overnight; (2) incubation in 50% methanol

for 30 min; (3) wash with H_2O milli-Q for 15 min (x 2); (4) incubation in 0.02% (w/v) sodium thiosulfate for 1 min; (5) wash with H_2O milli-Q for 1 min (x2); (6) incubation in 0.1% (w/v) silver nitrate for 30 min; (7) wash with H_2O milli-Q for 1 min (x3); (8) incubation in 0.04% (v/v) formaldehyde 2% (w/v) sodium nitrate until complete appearance of the spots; (9) incubation in 5% (v/v) acetic acid for 5 min to stop staining. Once stained, the gels were digitised with a GS-800 densitometer (Bio-Rad) and analysed with PD Quest V.7.4.0 software (Bio-Rad). Fig. IV.2 shows a scheme of the process of image analysis and comparison between geographic regions, which involved discarding the worst gel replicate from each clonal culture, thus using the best three gel replicates for the analysis. Highly reproducible replications were observed in gels of each clonal culture. The analysis of the gels was made region by region, producing a master gel of each region including just the spots shared by all the gels of the region; then the master gels of each region were compared to produce a final master gel in which spots shared by regions and spots exclusive of each region were discriminated. Percentages of similitude between gels were calculated as the number of common spots shared by two gels with regard to the total number of spots:

$$PS = [C \times 2 / (T1 + T2)] \times 100$$

where PS is the percentage of similitude, C is the number of the common spots shared by the 2 regions, and T1 and T2 are the total number of spots in region 1 and 2, respectively.

IV.3.4. Protein identification and database searching

Nineteen spots shared by gels of every region and 35 spots that were exclusive of one region were selected for protein identification. The number of spots selected for identification was limited by funding availability, thus the selection criterion was to choose the most intense spots among the common and the region exclusive ones. The selected spots were excised manually within a laminar flow cabin with sterile scalpel blade. Protein identification by LC-MS/MS analysis and *de novo* sequencing were carried out in the LP-CSIC/UAB Proteomics laboratory (Barcelona, Spain), member of ProteoRed network. Details on the LC_MS/MS procedure are provided as "Supplementary Materials and Methods" (Annex I). All sequence tags obtained from *de novo* sequencing were manually confirmed and were submitted to a homology search using the pBLAST algorithm (NCBI, USA). Data were contrasted against non-redundant NCBI (National Center for Biotechnology Information, Maryland, USA) database using Alveolata as search category. Theoretical Mr and pI values were obtained with the compute pI/Mw tool at the ExPASy Proteomics Server (http://www.expasy.org/tools/pi_tool.html).

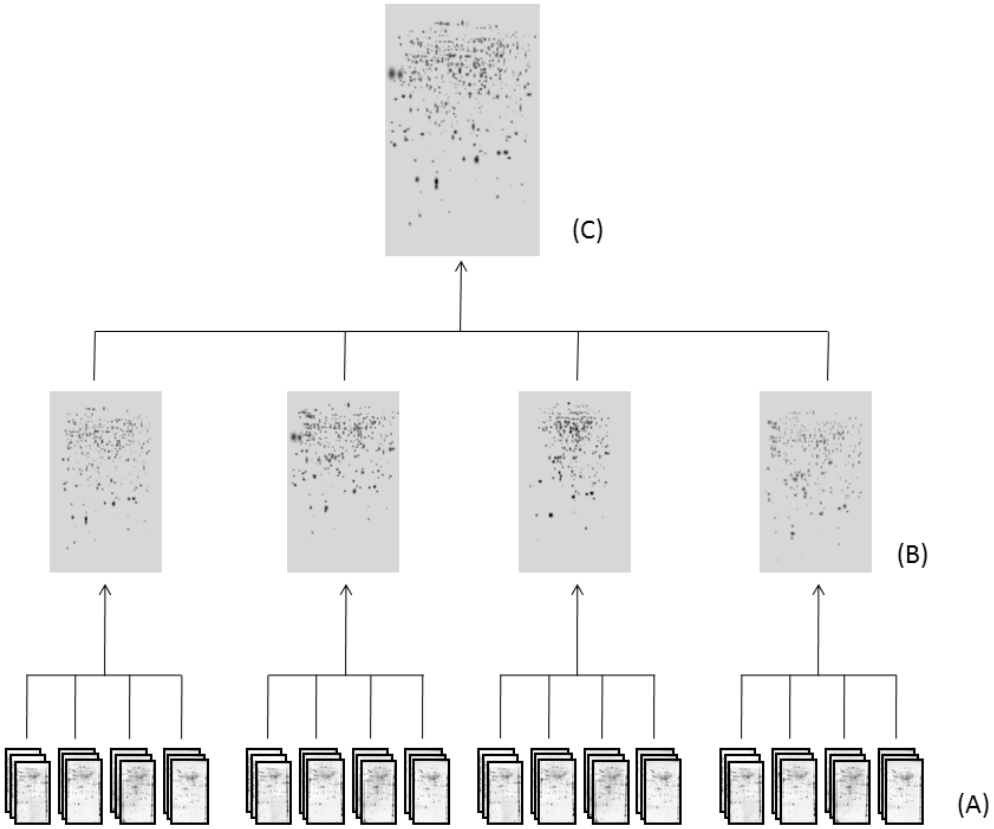

Fig. IV.2. Scheme of gel analysis and comparison between regions. (A) Analysis of four *Perkinsus olseni* clonal cultures by triplicate from each region to obtain a master gel of each region including just the spots shared by all the gels. (B) Comparison among master gels of each region. (C) Final master gel discriminating the spots shared by regions and the spots exclusive of each region.

IV.3.5. Statistical analysis

In vitro proliferation of each *P. olseni* clon was measured as the number of cells counted in the clonal cultures after two months from starting culture with $5x10^6$ cells. As explained above, 6 successive replicates of each clon were produced to harvest enough number of cells for proteomic analyses. Differences in *in vitro* proliferation were analysed by a two-factor nested ANOVA (geographical origin as main factor and clon nested under origin). The number of cells was root-squared transformed because of statistical requirements. As no significant effect due to clon was detected, the clones were grouped by origin and paired comparisons between origins were performed with Fisher's test. Statistical analyses were performed using MINITAB 16 statistical software. The significance level was established at $P \leq 0.05$.

IV.4. RESULTS

IV.4.1. In vitro *proliferation of* Perkinsus olseni *clones.*

Table IV.1 shows measures of *in vitro* proliferation of the four *P. olseni* clones from each geographic origin. No significant effect due to clon was detected but differences between origins were significant. The highest proliferation corresponded to *P. olseni* from Delta de l'Ebre and Carreras River and the lowest one to Ría de Arousa and Ría de Pontevedra, although differences between Ría de Pontevedra and Carreras River were not significant.

Table IV.1. *Perkinsus olseni in vitro* proliferation corresponding to 4 clonal cultures from each of 4 locations. Proliferation was measured as the number of cells counted in the cultures after two months from starting culture with $5x10^6$ cells. The mean values corresponding to 6 successive replicates performed for each clon are shown. Different letters above numbers denote significant differences.

Location	# Clon	Mean no. cells of each clon($x10^6$)	Mean no. cells of each location ($x10^6$)	Mean increment of no. cells (%)
Ría de Arousa	1	25.9	36.6^{a}	733
	2	39.4		
	3	49.5		
	4	31.7		
Ría de Pontevedra	1	39.2	46.1^{ab}	938
	2	51.8		
	3	43.5		
	4	49.8		
Delta de l'Ebre	1	68	64.3^{c}	1287
	2	63.1		
	3	64.4		
	4	62.0		
Carreras River	1	54.9	58.7^{bc}	1137
	2	65.3		
	3	57.3		
	4	57.1		

IV.4.2. Protein expression patterns of Perkinsus olseni

Fig. IV.3 shows representative 2-DE gels corresponding to *P. olseni* cultures from every region; high similitude between gels was observed. A total of 831 different spots were found in gels of Ría de Arousa, 730 in those of Ría de Pontevedra, 629 in Carreras River, and 660 in Delta de l'Ebre. Comparison of gels between clonal cultures within each population showed percentages of similitude higher than 70 % in each pair comparison except in 2 cases of Ría de Pontevedra; the highest mean percentage of similitude corresponded to Carreras River and the lowest to Ría de Pontevedra (Table IV.2).

A master gel for *P. olseni* from Ría de Arousa was built including the 417 spots shared by every gel of that region, the master gel of Ría de Pontevedra included 326 spots, that of Carreras River 346, and the one of Delta de l'Ebre 315. The comparison between master gels from every region showed that 141 spots were shared by all the regions; 39 (9%) spots of the master gel of Ría de Arousa were exclusive of that region (did not occur in the master gels of the other regions), 57 (17%) were exclusive of Ría de Pontevedra, 37 (11%) of Carreras River and 47 (15%) of Delta de l'Ebre. The percentages of similitude between regions (range: 55.5% - 71.9%; mean: 65.7; Table IV.3) were lower than those between clonal cultures within each region (range: 64.6% - 87.7%; mean: 80.1; Table IV.2). Higher percentages of similitude between *P. olseni* from the Atlantic regions (Carreras River, Ría de Arousa and Ría de Pontevedra) than between parasites from Delta de l'Ebre with those from Atlantic regions were observed (Table IV.3).

IV.4.3. Protein identification

A total of 54 spots, including 19 spots shared by *P. olseni* from all the regions and 35 spots exclusive of *P. olseni* from one population were excised for sequencing and identification. The percentage of shared spots that could be sequenced was higher than that of the exclusive spots; some of the sequenced spots did not significantly match with any protein of the screened databases (Table IV.4, Figs. IV.4 and IV.5). Table IV.5 shows the spots that were annotated.

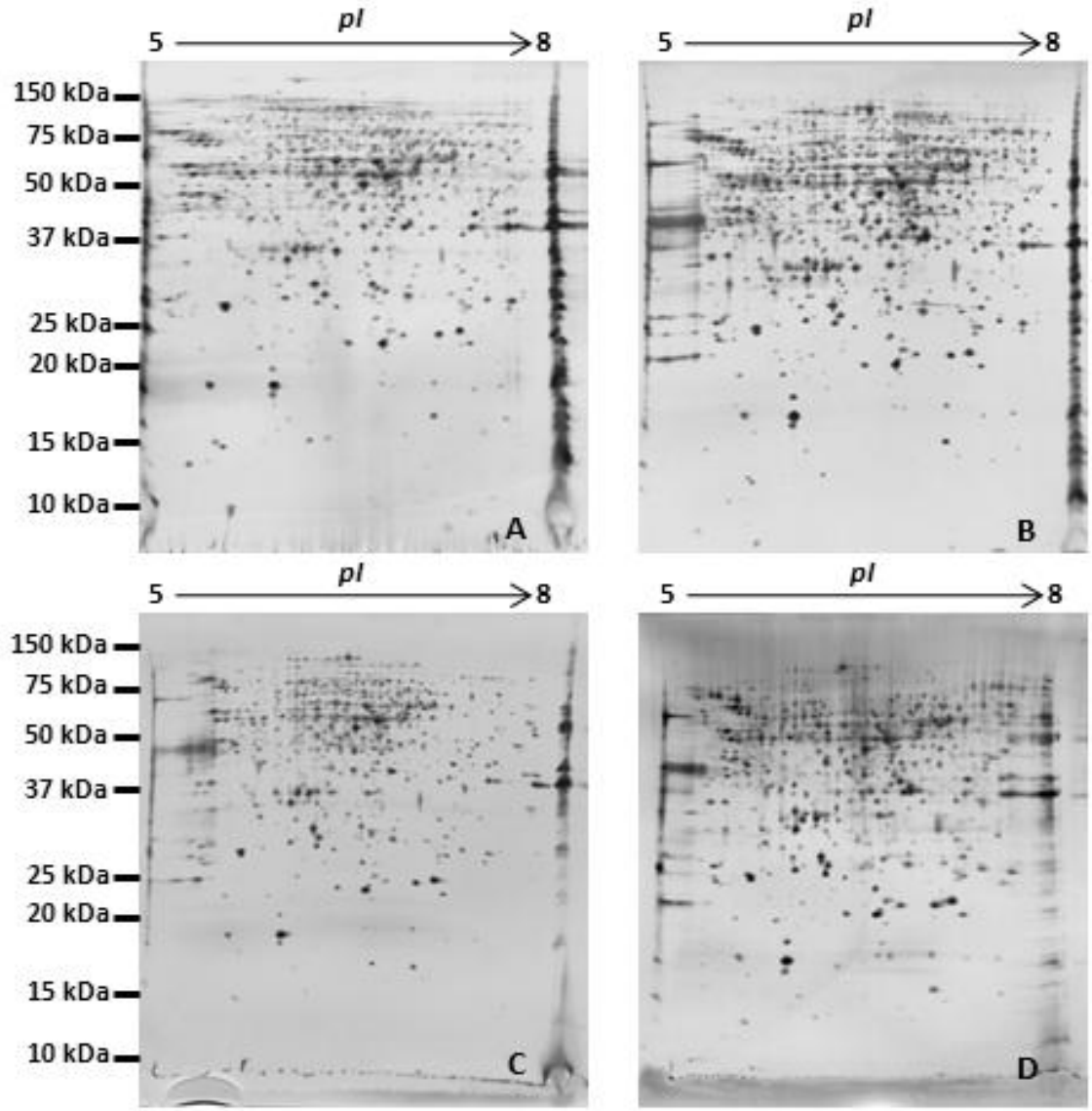

Fig. IV.3. Digitised images of silver-stained gels produced by 2D-SDS–PAGE of proteins extracted from *Perkinsus olseni* clonal cultures deriving from isolates from Ría de Arousa (A), Ría de Pontevedra (B), Delta de l'Ebre (C) and Carreras River (D).

Table IV.2. Comparison of protein expression patterns between *Perkinsus olseni* clonal cultures of the same geographical origin within each location. For each location, diagonal cells show the number of spots shared by the four gel replicates from each clonal culture,.the number of spots shared between pairs of clonal cultures is shown in cells below the diagonal, and the percentage of similitude between pairs of clonal cultures is shown in cells above the diagonal. The mean percentage of similitude of the paired comparisons within each location is also shown.

Location	**# Clon**					**Mean percentage of similitude**
	# Clon					
		1	2	3	4	
Ría de Arousa	1	623	84,6	79,7	84,5	81.1
	2	499	557	74,0	82,3	
	3	523	461	689	81,5	
	4	532	491	540	636	
		1	2	3	4	
Ría de Pontevedra	1	725	64,6	79,3	83,6	75.3
	2	352	364	77,5	66,3	
	3	496	345	526	80,6	
	4	582	342	481	668	
		1	2	3	4	
Delta de l´Ebre	1	490	87,7	79,7	77,9	80.6
	2	418	463	78,3	79,8	
	3	373	356	446	80,9	
	4	403	402	401	545	
		1	2	3	4	
Carreras River	1	437	83,1	85,6	81,7	83.5
	2	382	482	83,8	82,2	
	3	376	387	441	84,7	
	4	413	434	430	574	

Table IV.3. Number of spots shared between pairs of regions (below diagonal) and percentage of similitude between pairs of regions (above diagonal).

	Ría de Arousa	Ría de Pontevedra	Delta de l'Ebre	Carreras River
Ría de Arousa	-	68.9	63.7	71.9
Ría de Pontevedra	255	-	55.5	71.8
Delta de l'Ebre	231	179	-	62.2
Carreras River	271	242	205	-

Table IV.4. Total number of spots and number of spots excised, sequenced and annotated, corresponding to the group of spots shared by *Perkinsus olseni* from all the regions and the groups of spots exclusive of *P. olseni* from each region.

	Shared by all the regions	Exclusive from Ría de Arousa	Exclusive from Ría de Pontevedra	Exclusive from Carreras River	Exclusive from Delta de l'Ebre
Total	141	39	57	47	32
Excised	19	7	11	8	9
Sequenced	15	3	1	1	2
Annotated	13	3	1	1	1

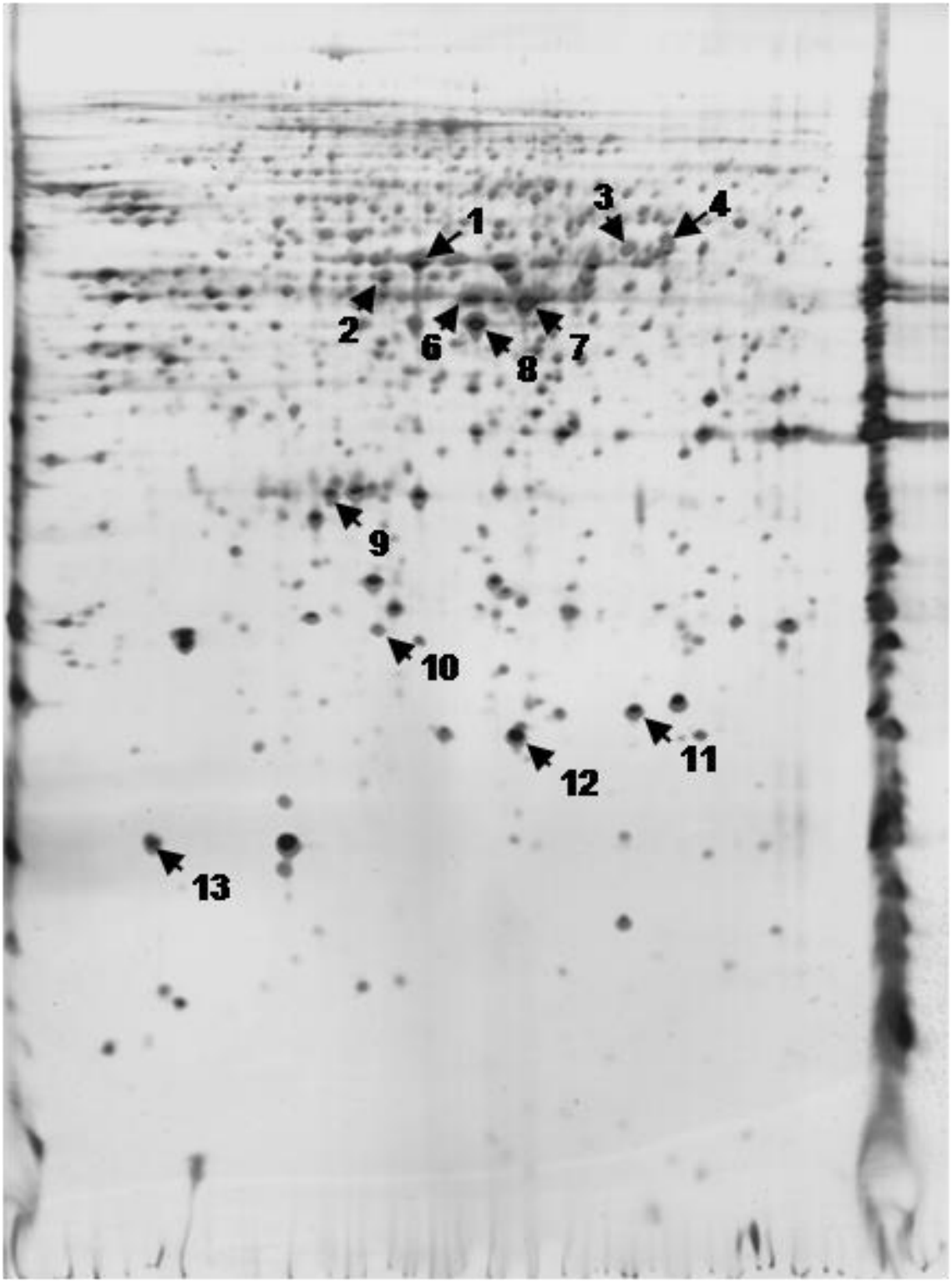

Fig. IV.4. Digitised image of a silver stained gel of proteins extracted from a *Perkinsus olseni* clonal culture deriving from Ría de Arousa. The spots that were annotated from those shared by parasites from all the regions are numbered in the image.

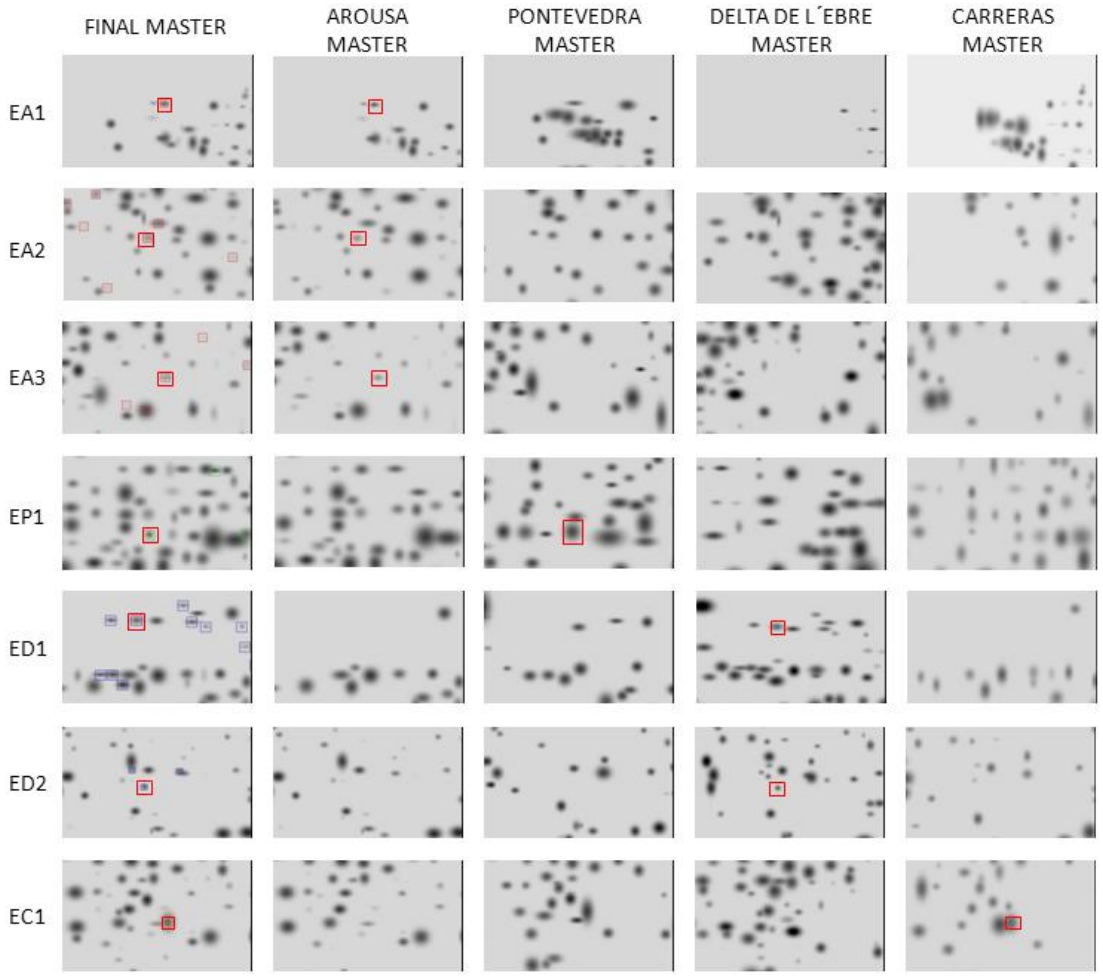

Fig. IV.5. Fragments of master gels showing the spots exclusive of *Perkinsus olseni* from one region that were annotated. Left column corresponds to the areas of final master gel where the exclusive spots occur. The remaining columns correspond to the same areas in the master gel of each region. Each file corresponds to an exclusive spot labelled with the spot code (the same as in Table IV.3). EA: exclusive of Ría de Arousa; EC: exclusive of Carreras River, ED: exclusive of Delta de l'Ebre; EP: exclusive of Ría de Pontevedra.

Table IV.5. Peptides annotated after searching in Blast database against Alveolata group in non-redundant proteins database. Spot codes 1-13 correspond to spots shared by *Perkinsus olseni* from all the regions, "EA" corresponds to spots exclusively found in *P. olseni* from Ría de Arousa, "EC" to spots exclusive from Carreras River, "ED" to spots exclusive from Delta de l'Ebre and "EP" to spots exclusive from Ría de Pontevedra. pI theo and pI obs: the theoretical (Swiss Prot, www.expasy.ch/tools) value of isoelectric point and the one observed in gels, respectively. Mw theor and Mw obs: the theoretical molecular weight and the one observed in gels, respectively. Identified protein: protein name of identified peptides in the database. Organism: the species to which corresponds the protein recorded in the database. A.no.: accession number of proteins in the database. Biological function: function of the protein according to Gene Ontology Biological Process. Peptide sequence: peptide sequences with the highest individual ion scores indicating identity or extensive homology ($p<0.05$). Sco: Score of the match of peptides with proteins in NCBI database after BLAST searching; the higher the value of the score, the better the peptide match. E-value: expected value, which describes the number of hits expected to see by chance when searching a database of a particular size; the lower the E-value, the better the peptide match

Spot code.	pI obs/ pI theor	Mw obs/ Mw theo (kDa)	Identified protein; organism	A. no.	Biological function	Peptide sequence	Sco	E value
1	6.25/6.00	62/56.55	Pyruvate kinase; *Perkinsus marinus*	XP_002788066.1	Glycolysis	VPSFQGTDHIIQSAINYGK	1080	0.0
2	6.15/8.35	54.5/17.08	Adenosylhomocysteinase; *P. marinus*	XP_002781556.1	One carbon metabolic process	NNAIVGNIGHFDNEIQmER	66.8	3,00 E-013
3	7.03/6.20	60/61.04	glucose-6-phosphate isomerase; *P. marinus*	XP_002787676.1	Glycolysis	FVAHIQQLDMESNGK	52.4	2,00 E-008
4	7.12/6.20	62/61.04	glucose-6-phosphate isomerase; *P. marinus*	XP_002787676.1	Glycolysis	FVAHIQQLDMESNGKR	55.8	2,00 E-009
5	7.12/6.04	54/47.33	Formate dehydrogenase ; *P. marinus*	XP_002782414.1	Oxidoreductase	DVEGMHLGTVA	32.5	0.057
6	6.50/5.23	49/48.63	Enolase 2; *P. marinus*	XP_002785647.1	Glycolysis	SGETEDTFIADIVVGLGTGQIK	70.2	2,00 E-014
7	6.70/5.23	50/48.63	Enolase 2; *P. marinus*	XP_002785647.1	Glycolysis	VNQIGSVTESIEANNK	52.4	1,00 E-010
8	6.50/5.82	48/44.77	Phosphoglycerate kinase 1; *P. marinus*	XP_002778580.1	Glycolysis	AGATSIIGGGDTAAmVEQQGK	65.5	2,00 E-012
9	6.05/5.58	32/25.05	Malate dehydrogenase; *P. marinus*	XP_002784022.1	Carbohydrate metabolic process	ImGLmSLDVTR	39.7	1.00 E-006
10	6.25/5.58	25/24.35	Conserved hypothetical protein; *P. marinus*	XP_002776039.1	Unknown	VEPATQP	21	0.76
11	7.05/5.86	22/21.55	Peroxiredoxin-2; *P. marinus*	XP_002765329.1	Oxidation-Reduction process / Antioxidant activity	VLDSIIETDEHGVVcPANWK	69.4	5,00 E-014

Spot code.	pI obs/ pI theor	Mw obs/ Mw theo (kDa)	Identified protein; organism	A. no.	Biological function	Peptide sequence	Sco	E value
12	6.5/6.03	20.5/22.11	Iron-dependent superoxide dismutase; *P. marinus*	XP_002788749.1	Superoxide metabolic process / Oxidation-Reduction process	MSAETLQYHYG	37.1	1.00 E-002
13	5.58/6.28	17.5/17.00	Peroxiredoxin V; *P. chesapeaki*	ABV22156.1	Oxidation-Reduction process / Antioxidant activity	ALGVDFDVTPVLGNVR	52.4	2,00 E-008
EA1	5.65/5.39	81/81.53	Heat Shock Protein 90; *P. marinus*	XP_002773238.1	Protein folding / Response to stress	LVNSPAVVTSAISPQMR	55.8	2,00 E-009
EA2	6.50/5.23	47/42.8	Galactokinase; *P. marinus*	XP_002788912.1	Galactose metabolic process	SGQLVHLDcR	35.4	0.005
EA3	7.28/5.51	41.5/141.49	Conserved hypothetical Protein; *P. marinus*	XP_002773742.1	Unknown	SLLDQDPENAEIK	44.3	9.00 E-006
EP1	6.15/5.42	63.1/56.36	Pyruvate kinase; *P. marinus*	XP_002788067.1	Glycolysis	HVAVMLDTK	32.5	0.038
ED1	6.8/5.71	100/30.55	Malate dehydrogenase; *P. marinus*	XP_002787252.1	Malate metabolic process	LSLYVAcAGINPGR	46.9	1,00 E-006
EC1	6.85/5.79	36.5/36.38	Glyceraldehyde 3 phosphate dehydrogenase; *P. marinus*	XP_002788755.1	Oxidation/Reduction process	VIISAPPKDDTPMFVMGVNNK	72.3	7.00 E-015

IV.5. DISCUSSION

The results showed that there is a remarkable variability in the protein expression of *in vitro* cultured *P. olseni* cells depending on the geographic origin. Genetic and physiological variability depending on geographic origin had also been found in *P. marinus* (Reece et al., 1997; Kotob et al., 1999; Reece et al., 2001; Thompson et al., 2011). The variability between regions was higher than between *P. olseni* clones within each region; the percentages of similitude between clones from the same region was high (above 80% on average) in 3 locations and slightly lower in the other one. The number of spots shared by *P. olseni* from all the regions was higher than the numbers of spots exclusive of *P. olseni* from each region; this result contrasts with the comparison of the protein expression between 3 *Perkinsus* spp. (*P. olseni*, *P. marinus* and *P. chesapeaki*), in which the numbers of spots exclusive of each species was much higher than the number of spots shared by the 3 species (Chapter VI); lower variability between clones from the same species than between clones from different species was expected. Higher similarity was found among the proteome of *P. olseni* from the Atlantic regions than between the Mediterranean location and the Atlantic ones. Nevertheless, the highest similarity did not occur between the two closest locations (Arousa and Pontevedra) but between Carreras River and the other two Atlantic locations. This could be due to the fact that the movement of clams from the SW region of the Iberian Peninsula (Algarve and Huelva) to be immersed or depurated in the NW (Galicia) is much more frequent than movements of clams from NE (Catalonia) to the SW and NW or from the NW to the NE. Considering that the host source (*R. decussatus*) of the *P. olseni* isolates from the Atlantic locations was a different species from the host source (*R: philippinarum*) of the isolates from the Mediterranean location, to what extent the host difference influenced the variability remains unknown. Differences in *P. olseni in vitro* proliferation due to geographic origin were also detected, it was lower in clones from Ría de Arousa and Ría de Pontevedra and higher in Delta del'Ebre and Carreras river, which could be the result of local adaptation to environmental conditions; interestingly, water is warmer during summer in the two latter locations. Previous chapter using microsatellites to analyse genetic variability of *P. olseni* among the same locations as in this chapter showed no correlation between geographic and genetic distances.

In the case of the spots shared by *P. olseni* from all the regions, a high proportion of the annotated proteins were involved in sugar associated metabolism, which could be due to the fact that the parasite cells were collected at the culture exponential growth phase. The occurrence of more than one spot in gels corresponding to glucose 6-P isomerase and enolase-2 suggests that several isoforms of those proteins occur in the parasite. In the case of glucose 6-P isomerase gene, different alleles were found in the oomycete *Phytophthora infestans* (Ospina-Giraldo

and Jones, 2003) as well as in *Entamoeba histolytica* (Razmjou et al., 2006). In the apicomplexan parasite *Toxoplasma gondii,* which develops metabolic and morphological changes during the infection, several stage-specific isoenzymes were found; these isoenzymes have different biochemical properties that maintain the major parasitic metabolism such as glycolysis in pace with the stage-specific requirements of carbohydrate or polysaccharide biosynthesis, including isoforms of glucose 6-P isomerase and enolase-2 (Tomavo, 2001). In *P. olseni*, the isoforms could have similar functions as in *Toxoplasma gondii* regulating the metabolism according to the progress of the infection. The strictly conserved plant enolase pentapeptide EWGWC insertion that had been found in *P. marinus* (Joseph et al., 2010) was also found in *P. olseni* enolase-2 in this study. This domain was found previously in other apicomplexan parasites as *Plasmodium falciparum* (Read et al., 1994) and *Toxoplasma gondii* (Tomavo, 2001). Glucose 6-P isomerase and pyruvate kinase were proposed as antiparasite drug targets in humans (Rigden et al., 1999; Tomavo, 2001; Muñoz and Ponce, 2003). A better understanding of these proteins in *Perkinsus* would help to find tools to fight the disease. Perkinsozoa group, where the genus *Perkinsus* is included, is considered the earliest group diverging from the lineage leading to dinoflagellates, branching close to the node shared by dinoflagellates and apicomplexans (Saldarriaga et al., 2003; Bachvaroff et al., 2011; Fernández Robledo et al., 2011). Members of both dinoflagellates and apicomplexans as well as *P. olseni* possess plastids and recent analysis of newly identified photosynthetic members of the apicomplexan lineage have shown that these plastids evolved from a single common secondary endosymbiosis with a red alga (Teles-Grilo et al., 2007; Moore et al., 2008; Janouskovec et al., 2010; Fernández Robledo et al., 2011). This suggests that non-photosynthetic relatives of both lineages, including *Perkinsus*, evolved from photosynthetic ancestors, raising the possibility that these lineages retain cryptic organelles (Keeling, 2010). This common lineage ancestor could explain the existence of proteins derived from a plant origin as these enolase-2 as well as superoxide dismutase and peroxiredoxin proteins that are discussed next.

Enzymes linked to protection from host immune reaction were also well represented in the group of spots shared by all the regions that were annotated. A high constitutive expression of antioxidant proteins was detected. Proteins like peroxiredoxin (Prx) II and V and superoxide dismutase (SOD) were expressed in large quantities which suggests the importance of these proteins as defence mechanism. Their detoxifying role seems essential to infect the host and elude host defences; *P. marinus* inhibits the production of reactive oxygen intermediates when it is phagocytosed by host haemocytes, thus preventing oxidative damage (Robledo et al., 2008). In the case of the immune reaction of venerid clams against *P. olseni*, clam haemocytes encapsulate the parasite (Montes et al., 1995a; Villalba et al., 2004) and, according to our results, *P. olseni* also produces enzymes allowing to neutralise the reactive oxygen intermediates released from host haemocytes.

The S-adenosylhomocysteinase is a cytosolic enzyme that has important functions in the cytosolic metabolism linking processes of transmethylation, transsulfuration and purine metabolism (Kloor et al., 2000); the enzyme cleaves S-adenosylhomocysteine and provides homocysteine for the synthesis of cysteine and the regeneration of methionine (Takata and Fujioka, 1983). Methionine is also the source of cysteine, the limiting reagent for the synthesis of glutathione (GSH), one of the most important antioxidants in organisms (Company et al., 2011). The S-adenosylhomocysteinase was proposed as a target for chemotherapy in other protozoan parasites as *Trichomonas vaginalis* (Bagnara et al., 1996).

In the case of spots exclusive of one region, the annotated proteins were classified into four categories according to Gene Onthology, metabolism (galactokinase in Ría de Arousa and malate dehydrogenase in Delta de l'Ebre), glycolysis (pyruvate kinase in Ría de Pontevedra), response to stress (heat shock protein 90 in Ría de Arousa), and oxidation/reduction (glyceraldehyde 3-P dehydogenase in Carreras River). All these proteins are involved in processes related to the Kreb´s cycle except the heat shock protein 90. Malate dehydrogenase, which was found in common proteins as well as Delta de l´Ebre specific protein,is involved in the metabolism of the intermediaries of the Kreb´scycle; the former catalyses the transformation of L-malate to pyruvate together with the coenzyme $NADP^+$ (Sánchez et al., 1996). Pyruvate kinase catalyses the transformation of phosphoenolpyruvate to pyruvate, the previous step to the generation of Acetyl CoA. Two pyruvate kinases were detected in *P. olseni* from Ría de Pontevedra, the one shared by all the regions and one exclusive of this region. This specific protein from Ría de Pontevedra population seems to be the same proteins but with a posttranslational modification which rise its *pI* in 0.1 units. Galactokinase takes part in the Leloir route involving the transformation of galactose to glucose; it is an ATP-dependent enzyme catalysing the phosphorylation of galactose to form galactose-1-phosphate (Chu et al., 2009). Glyceraldehyde 3-P dehydrogenase is a key enzyme in glycolysis and catalyses the oxidative phosphorilation of glyceraldehyde-3P into 1,3-bisphosphoglycerate in the presence of NAD^+ and inorganic phosphate (Daubenberger et al., 2000). Heat shock protein 90 had been studied in *P. marinus* due to its involvement in the infection progression; this protein is necessary for the adaptation of the parasite to the host environment. *P. marinus,* as many other organisms uses heat shock proteins as part of its adaptive survival repertoire (Tirard et al., 1995).

Contrasting with the spots shared by *P. olseni* from all the regions, none of the annotated spots that were exclusively found in one region could be considered as directly linked to virulence, which would have supported the occurrence of virulence differences between *P. olseni* geographic strains; however, the detected differences (proteins involved in metabolism and stress response) could offer a better adaptation to environmental local conditions that would favour cell division / growth and thus possibly pathogenicity. Local adaptation of *Perkinsus spp*. to different ranges of salinity

and temperature can explain the adaptability to new environments, their proliferation and virulence (La Peyre et al., 2008; Thompson et al., 2014). Obviously, sequencing more spots would have allowed deeper analysis of *P. olseni* variability, but limited funding impeded going further.

V. VARIABILITY OF PROTEIN EXPRESSION PROFILING IN THE EXTRACELLULAR PRODUCTS OF *Perkinsus olseni* AMONG REGIONS OF THE SPANISH COAST

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V.1. ABSTRACT

The variability of the protein expression profiling in the extracellular products (ECPs) of in vitro cultured *Perkinsus olseni* deriving from 4 regions of the Spanish coast was evaluated. The regions involved were the rías of Arousa and Pontevedra (Galicia, NW Spain), Carreras River (Andalusia, SW Spain) and Delta de l'Ebre (Catalonia, NE Spain). *P. olseni in vitro* clonal cultures were produced from parasite isolates from each of four clams from each region. Proteins released by the *in vitro* cultured parasites were isolated and separated by two dimensional electrophoresis (2DE). Qualitative comparison of protein expression profiles in the *P. olseni* ECPs among regions was performed with PD Quest software. Around 130 spots were counted in the gels from ECPs of *P. olseni* clones from each region, of which 23 spots were shared by all the regions and various spots were exclusive of one region. A total of 34 spots were excised from the gels and analysed for sequencing. The protein cathepsin B, involved in proteolysis, the signal recognition particle receptor subunit β, involved in protein transport through membranes, and a protein belonging to N-acetyl transferase superfamily, involved in biosynthesis, were identified in spots shared by *P. olseni* ECPs from all regions. Pepsin A precursor, involved in proteolysis; heat shock protein (HSP) 60; and phosphoserin aminotransferase, involved in biosynthesis, were exclusively identified in *P. olseni* ECPs from Ría de Arousa, while peroxiredoxin V, involved in oxidation-reduction, was exclusively identified in *P. olseni* ECPs from Ría de Pontevedra. Differences in released proteins suggest different virulence or resistance to host attack between parasites from different locations.

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V.2. INTRODUCTION

The genus *Perkinsus* includes protistan parasites infecting molluscs over the world (Villalba et al. 2004, 2011). The widest spread species is *Perkinsus olseni* (Lester and Davis, 1981), which is associated with mass mortalities of carpet-shell clams *Ruditapes decussatus* in Portugal (Azevedo, 1989) and Spain (Sagristà et al., 1995) and of Manila clams *R. philippinarum* in Spain (Sagristà et al., 1995), Italy (Pretto et al. 2014), Korea (Park and Choi, 2001), Japan (Choi et al., 2002) and China (Liang et al., 2001; Wu et al., 2011). *Perkinsus marinus* has affected American oyster *Crassostrea virginica* populations in USA for more than 50 years devastating the production (Burreson and Ragone-Calvo, 1996; Soniat, 1996). Both *P. olseni* and *P. marinus* are included in the list of notifiable diseases of the World Organisation for Animal Health (http://www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2014/).

The importance of the extra cellular products (ECPs) of *P. marinus* in its infectivity was highlighted in previous studies (La Peyre et al., 1995; Garreis et al., 1996; Tall et al., 1999). *P. marinus* releases serine proteases that can degrade laminin and fibronectin, which are the major extracellular matrix component of stroma and basement membrane; these serine proteases can affect host immune ability by reducing oyster haemocyte mobility and lysosomal activity in oyster haemolymph (Garreis et al., 1996). The reduction of oyster haemocyte mobility due to the infection with *P. marinus* suppress the vibriocidal activity of oyster haemocytes to effectively eliminate *V. vulnificus* (Tall et al., 1999). The progression of *P. marinus* infection is inhibited by bacitracin, a protease inhibitor, which supports considering *P. marinus* ECP proteases as virulence factors (Faisal et al., 1999). A N-glycosylated serine protease occurring in ECPs was isolated and characterised; this 41.7 kDa monomeric protease was designed as perkinsin, the major extracellular protease produced by *P. marinus in vitro* (La Peyre et al., 1995b; Faisal et al., 1999). A serine protease gene with a sequence closer to family subtisilin was identified in the *P. marinus* genome (Brown and Reece, 2003). Besides serine proteases, high levels of acid phosphatase were found in *P. marinus* ECPs; this enzyme could dephosphorilate enzymes as NADPH oxidase, which blocks the production of reactive oxygen species (ROS) by the host (Volety and Chu, 1997). These findings led to test the occurrence of 19 hydrolytic activities in the ECPs of four *Perkinsus* spp. (*P. marinus*, *P. olseni*, *P. chesapeaki* and *P. mediterraneus*) using commercial API ZYM® kits (BioMérieux); most of these enzymes have been detected in the four species but their relative concentrations appear to be unique to each *Perkinsus* species. Thus, *P. marinus* culture supernatants showed high α-chymotrypsin activity (non-detected in *P. olseni* and *P. chesapeaki*); those of *P. olseni* high esterase, esterase lipase, and α-mannosidase; *P. chesapeaki* high alkaline phosphatase; and *P. mediterraneus* high naphtol-AS-BI-phosphohydrolase and acid phosphatase activities; α-glucosidase activity was high in the four species (Casas et al., 2002b, 2008, 2009).

Furthermore, gelatine SDS-PAGE demonstrated proteolytic activity in various bands from *P. mediterraneus* and *P. chesapeaki* ECPs (Casas et al., 2008, 2009).

Variability in virulence and other physiological aspects through populations of *P. marinus* has been demonstrated (La Peyre et al., 1995; Bushek and Allen, 1996; Chu and Lund, 2006; Alemán Resto and Fernández Robledo, 2014), which is consistent with the occurrence of genetic variability among geographic strains (Reece et al., 1997; Reece et al., 2001; Thompson et al., 2011; 2014a, 2014b). Knowing differences in virulence is important to avoid host movements from areas with high virulent strains to areas with less virulent strains. Variability of *P. olseni* populations through the Spanish coast has been addressed using genetic markers (Chapter III) and based on the parasite proteome (Chapter IV). Evaluating the variability of the protein expression profiles in ECPs of *P. olseni* was planned as a way to assess variability of virulence.

Proteomic approaches have been used to investigate secreted proteins of both intra- and extra-cellular parasites such as *Plasmodium falciparum* (Vincensini et al., 2005), *Trichomonas vaginalis* (Kucknoor et al., 2007), *Giardia Lambia* (Ringqvist et al., 2008) and *Leishmania (Viannia) braziliensis* (Cuervo et al., 2009), with high success in the protein identification. These studies led to identify several proteins involved in host-parasite interaction, modulation of the immune system of the host and host cell signalling (Cuervo et al., 2009). The aim of this study was to identify the most abundant proteins included in ECPs of *P. olseni* and to evaluate variability of the protein expression profiles in ECPs through the Spanish coast, with a proteomic approach combining two dimensional electrophoresis (2DE) and mass spectrometry liquid chromatography-tandem mass spectrometry (LC-MS/MS and de novo sequencing).

V.3. MATERIALS AND METHODS

V.3.1. Production of in vitro *clonal cultures*

Parasites *Perkinsus olseni* were isolated from 4 carpet shell clams *R. decussatus* from Ría de Arousa, 4 from Ría de Pontevedra in Galicia (NW Spain), 4 from Carreras River in Huelva (Andalucía, SW Spain) and from 4 Manila clams *R. philippinarum* from Delta de l´Ebre in Catalonia (NE Spain) (Fig.V.1). The parasites were isolated from the gills and allowed to proliferate *in vitro*; the in vitro cultures (one per host) were cloned as described in chapter IV, section 2.1. One monoclonal derivate of each isolate was *in vitro* expanded and used in the study. Culture medium was separated from cells after centrifugation (800 x g, 10 min, 25 °C) and it was lyophilised and stored at -80ºC until used. The process was repeated until obtaining 150 mL culture medium, a volume previously estimated as suitable to get enough protein to perform 2DE.

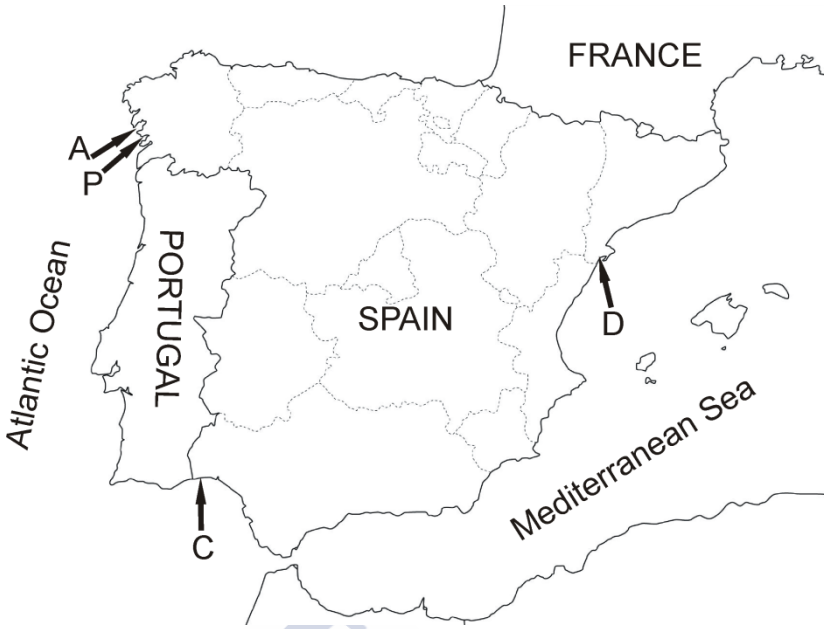

Fig. V.1. Map showing the four Spanish locations where infected clams were collected: Ría de Arousa (A) and Ría de Pontevedra (P) in Galicia, Carreras River (C) in Huelva (Andalusia) and Delta de l'Ebre (D) in Catalonia.

V.3.2. Protein extraction

Culture medium samples were resuspended in 4 mL of lysis buffer (8M Urea, 2M Thiourea, 2% CHAPS , 1% DTT, 0.8% Ampholites, and 100mM phenylmethylsulphonyl fluoride, in order to avoid the protease activity in the sample) for 2.5 h at 4 °C and centrifuged at 12000 x g for 30 min to extract the ECP proteins. The protein concentration was determined by Lowry assay using the RC/DC Protein Assay Bio-Rad and measuring in a microplate lecture Expert 96 (Asys Hitech).

V.3.3. Two dimensional electrophoresis and imaging analysis

Preliminary trials had been performed to tune up the experimental conditions for separation of *P. olseni* extracellular proteins by 2DE. Each sample was processed in quadruplicate. For the first dimension, aliquots of 100 µg of protein were diluted to a final volume of 250 µL in rehydration solution. Samples were centrifuged for 1 min at maximum speed to remove bubbles and loaded onto the immobilised pH gradient (IPG)-strips (11cm, pH 5-8, Bio-Rad) by in-gel rehydration. IEF was performed in a horizontal apparatus (Protean®IEF System from Bio-Rad) at 20ºC as follows: 50 V was applied during the rehydration step during 12 hours, the IEF was started with 250 V for 15 min, 8000 V for 150 min and 8000 V until 35000 Vh. After IEF, the strips were incubated for 20 min in equilibration buffer (50 mM Tris–HCl pH 8.8, 6M urea, 30% glycerol, 2% SDS) with 10 mg/ml DTT to reduce the proteins and, in a second step, incubated for 20 min in this mix with iodoacetamide at 45 mg/ml to alkylate proteins.

The second dimension involved separation in SDS-PAGE, which was performed through 12,5% polyacrylamide homemade gels, using a Protean®II xi cell (Bio-Rad), as described in chapter IV, section 2.3. Four gels were produced from each clonal culture. The gels were silver stained with a protocol compatible with MS analysis as described chapter IV, section 2.3. Once stained, the gels were digitised with a GS-800 densitometer (Bio-Rad) and analysed with PD Quest V.7.4.0 software (Bio-Rad). Fig. V.2 shows a scheme of the process of image analysis and comparison between geographic regions, which involved discarding the worst gel replicate from each clonal culture, thus using the best three gel replicates for the analysis. Highly reproducible replications were observed in gels of each clonal culture. The analysis of the gels was made region by region, producing a master gel of each region including just the spots shared by all the gels of the region; then the master gels of each region were compared to produce a final master gel in which spots shared by regions and spots exclusive of each region were discriminated. Percentages of similitude between gels were calculated as the number of common spots shared by two gels with regard to the total number of spots:

$$PS = [C \times 2 / (T1 + T2)] \times 100$$

where PS is the percentage of similitude, C is the number of the common spots shared by the 2 regions, and T1 and T2 are the total number of spots in region 1 and 2, respectively.

V.3.4. Protein identification and database searching

Eleven spots shared by gels of every region and 23 spots that were exclusive of one region were selected for protein identification. The number of spots selected for identification was limited by funding availability, thus the selection criterion was to choose the most intense spots among the common and the region exclusive ones. The selected spots were excised manually within a laminar flow cabin with sterile scalped blade. Protein identification by LC-MS/MS analysis was carried out in the LP-CSIC/UAB laboratory, a member of ProteoRed network. All sequence tags obtained from *de novo* sequencing were manually confirmed and were submitted to a homology search using the pBLAST algorithm (NCBI, USA). Data were contrasted against non-redundant NCBI (National Center for Biotechnology Information, Maryland, USA) database using Alveolata group as search category. Theoretical Mw and pI values were obtained with the compute pI/Mw tool at the ExPASy Proteomics Server (http://www.expasy.org/ tools/ pi_tool.html).

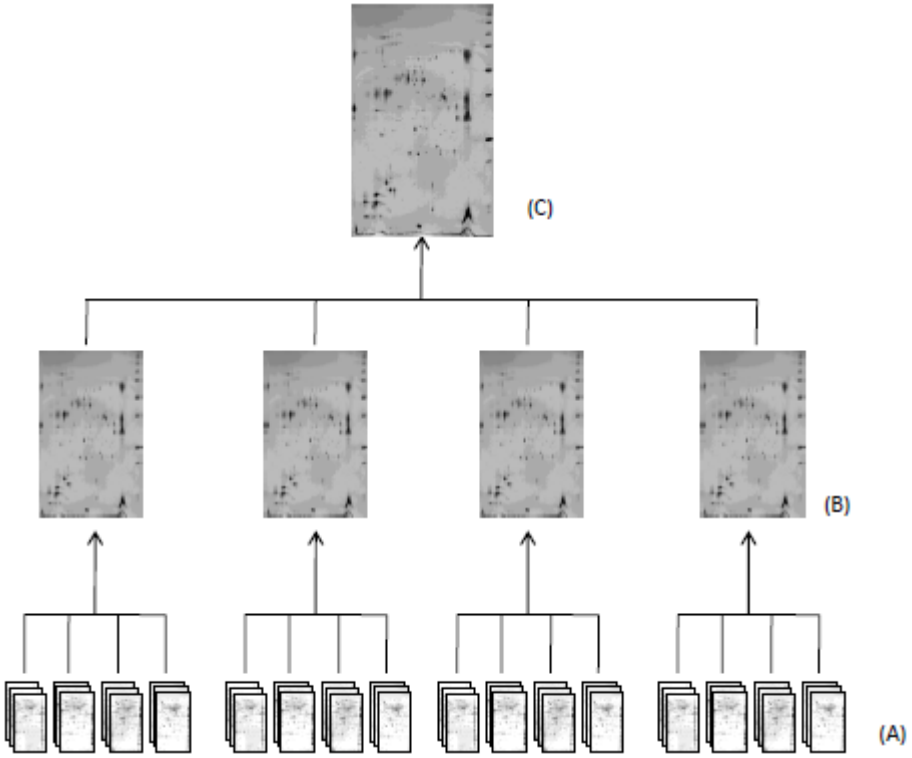

Fig. V.2. Scheme of gel analysis and comparison between regions. (A) Analysis of four *Perkinsus olseni* clonal cultures by triplicate from each region to obtain a master gel of each region including just the spots shared by all the gels. (B) Comparison among master gels of each region. (C) Final master gel discriminating the spots shared by regions and the spots exclusive of each region.

V.4. RESULTS

V.4.1. Protein expression patterns of Perkinsus olseni *extracellular products*

Fig. V.3 shows representative 2-DE gels corresponding to *P. olseni* extracellular products (ECPs) from every region; high similitude between gels was observed. A total of 118 different spots were found in gels of Ría de Arousa, 130 in those of Ría de Pontevedra, 132 in Carreras River, and 144 in Delta de l´Ebre. Comparison of gels between clonal cultures within each region showed percentages of similitude higher than 60% in each pair comparison except comparisons with clonal culture 1 of Ría de Arousa, which had much fewer spots shared in its replicates than the other clones; the highest mean percentage of similitude corresponded to Delta de l´Ebre and the lowest in Ría de Arousa (Table V.1).

Table V.1. Comparison of protein expression profiling between extracellular products of *Perkinsus olseni* clonal cultures from the same geographic origin. For each location, diagonal cells show the number of spots shared by the four gel replicates from each clonal culture. The number of spots shared between pairs of clonal cultures is shown in cells below the diagonal, and the percentage of similitude between pairs of clonal cultures is shown in cells above the diagonal. The mean percentage of similitude of the paired comparisons within each location is also shown.

Location	# Clon	# Clon				Mean percentage of similitude
		1	2	3	4	
Ría de Arousa	1	71	53.3	57.6	56.3	64.18
	2	56	139	76.1	73.6	
	3	53	113	158	68.2	
	4	49	89	89	103	
		1	2	3	4	
Ría de Pontevedra	1	135	90.8	87.6	77.4	82.61
	2	119	127	85.1	77.4	
	3	124	117	148	77.3	
	4	95	91	99	108	
		1	2	3	4	
Delta de l´Ebre	1	176	84.2	84.8	79.5	83.55
	2	133	140	84.3	85.8	
	3	134	118	140	82.8	
	4	118	112	108	121	
		1	2	3	4	
Carreras river	1	158	73.4	66.4	80.7	70.31
	2	102	120	68.0	69.9	
	3	80	69	83	63.5	
	4	132	101	80	169	

A master gel for *P. olseni* ECPs from Ría de Arousa was built including the 43 spots shared by every gel of that region, the master gel of Ría de Pontevedra included 86 spots, that of Carreras river 67, and the one of Delta de l´Ebre 144. The comparison between master gels from every region showed that 21 spots were shared by all regions; 5 spots (11.6%) of the master gel of Ría de Arousa were exclusive of that region (did not occur in the master gels of the other regions), 14 (16.3%) were exclusive of Ría de Pontevedra, 4 (6.0%) of Carreras river and 21 (14.6%) of Delta de l´Ebre. The percentages of similitude between regions (range: 43.6% – 72.5%; mean: 57.90%; Table V.2) were lower than those between clonal cultures within each region

(range: 64.18% - 83.55%; mean: 75.16%; Table V.1). Higher percentages of similitude between *P. olseni* from Delta de l´Ebre with Ría de Pontevedra and Carreras River than between parasites from Ría de Arousa with the other geographic origins were observed (Table V.2).

V.4.2. Protein identification

A total of 34 spots, including 11 spots shared by all populations and 23 spots exclusive from one population were excised for sequencing and identification. Ten out of 11 spots shared by *P. olseni* from all the regions that had been excised were successfully sequenced while 7 out of 23 excised spots exclusive from one region were sequenced (Table V.3). Some of the sequenced spots did not significantly match with any protein of the screened databases (Table V.3, Figs. V.4 and V.5). Table V.4 shows protein annotations.

Table V.2. Number of spots shared between pairs of locations (above diagonal) and percentage of similitude between pairs of locations (below diagonal).

	Ría de Arousa	Ría de Pontevedra	Delta de l´Ebre	Carreras River
Ría de Arousa	-	55.8	43.5	43.6
Ría de Pontevedra	36	-	70.5	61.4
Delta de l´Ebre	32	67	-	72.5
Carreras River	24	47	62	-

Table V.3. Total number of spots and number of spots excised, sequenced and annotated, corresponding to the group of spots shared by extracellular products (ECPs) of *Perkinsus olseni* from all the regions and the groups of spots exclusive of (ECPs) of *P. olseni* from each region.

	Shared by all the regions	Exclusive from Ría de Arousa	Exclusive from Ría de Pontevedra	Exclusive from Delta de l´Ebre	Exclusive from Carreras river
Total	23	5	14	21	4
Excised	11	4	7	10	2
Sequenced	10	4	2	1	0
Annotated	5	4	1	0	0

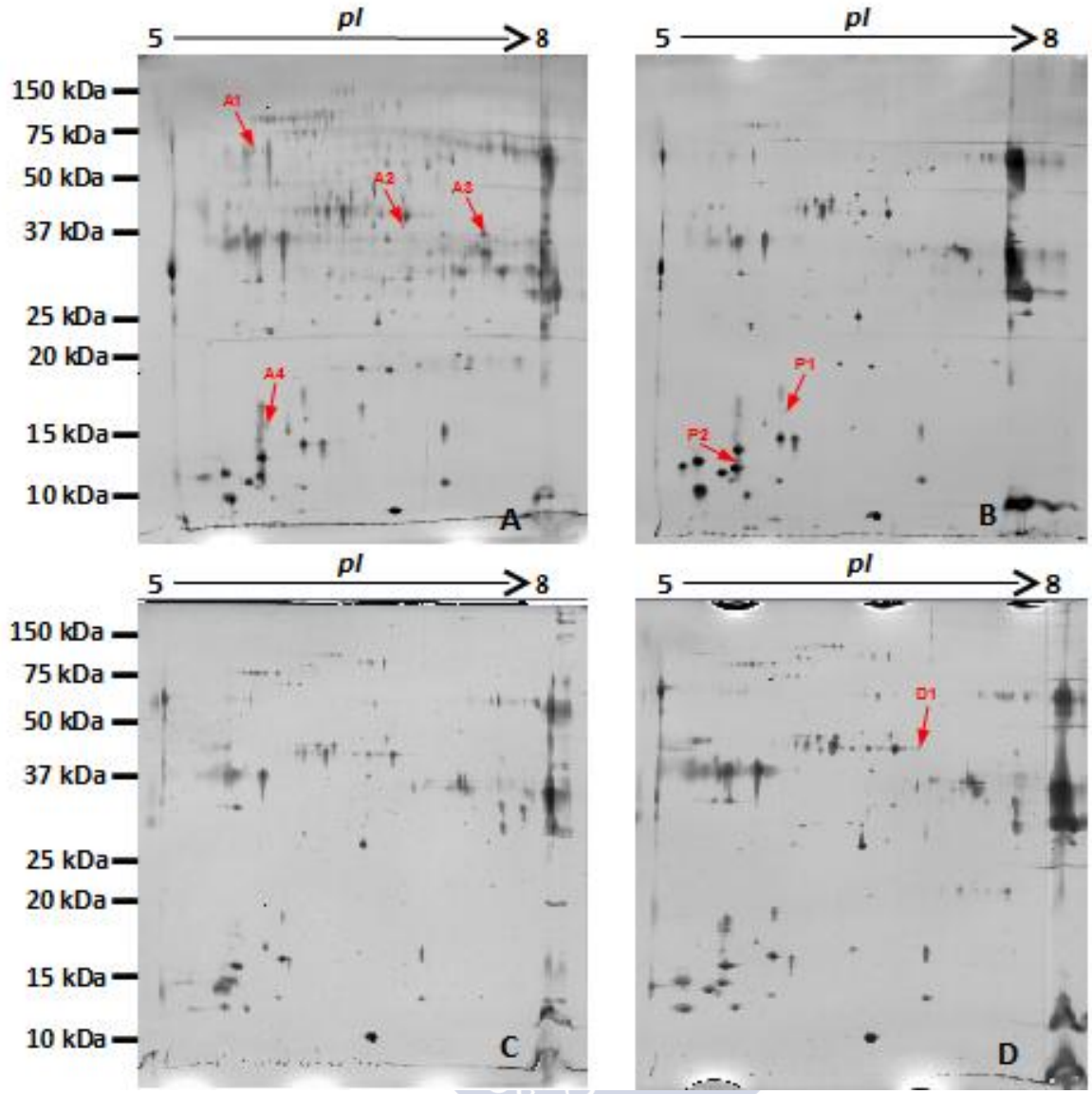

Fig. V.3. Digitised images of silver-stained gels produced by 2DE-PAGE of proteins occurring in extracellular products of *Perkinsus olseni* clonal cultures deriving from isolates from Ría de Arousa (A), Ría de Pontevedra (B), Delta de l'Ebre (C) and Carreras River (D).

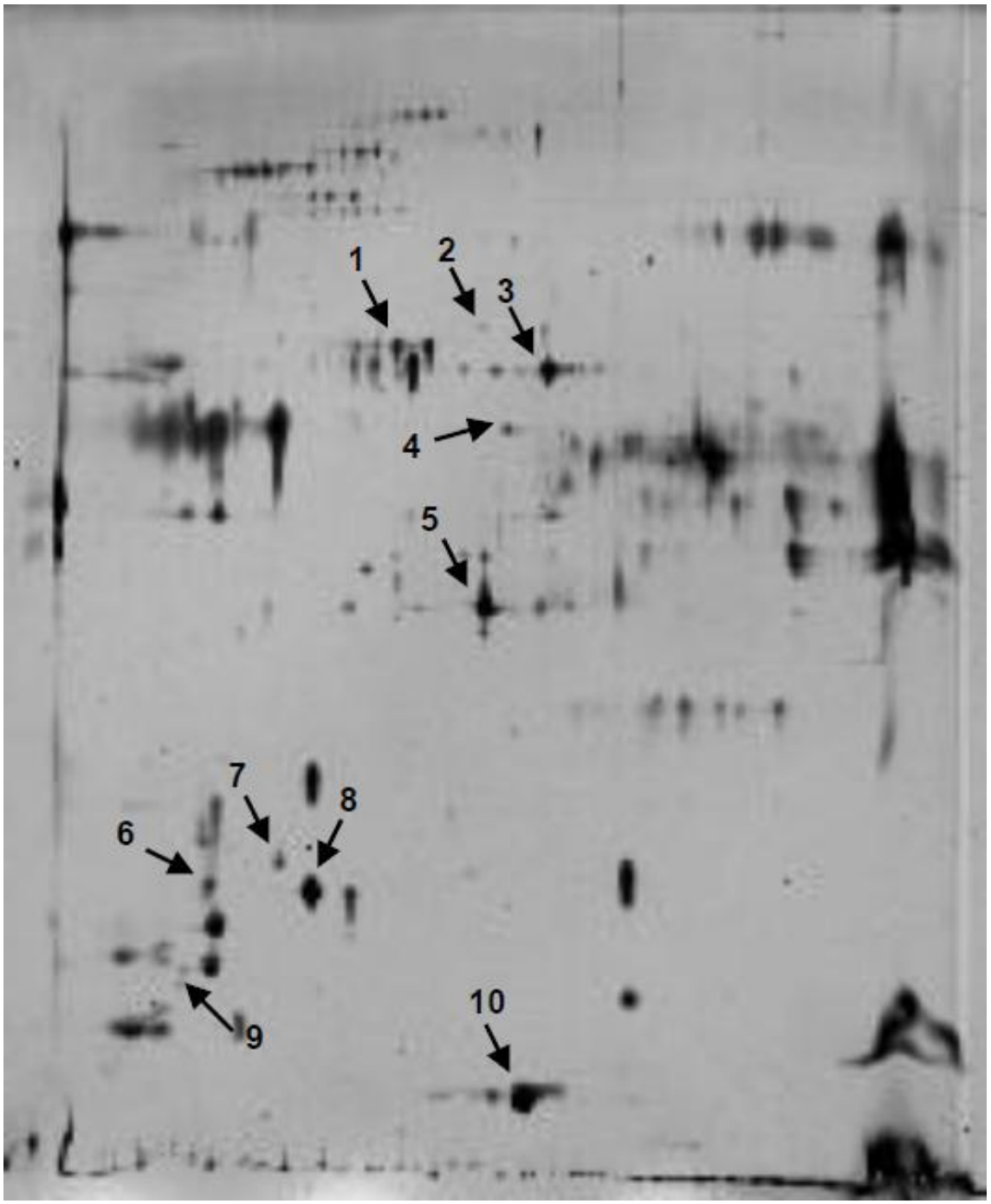

Fig. V.4. Digitised image of a silver stained gel of proteins occurring in extracellular products of a *Perkinsus olseni* clonal culture deriving from Delta de l´Ebre. The spots that were annotated from those shared by parasites from all the regions are numbered in the image.

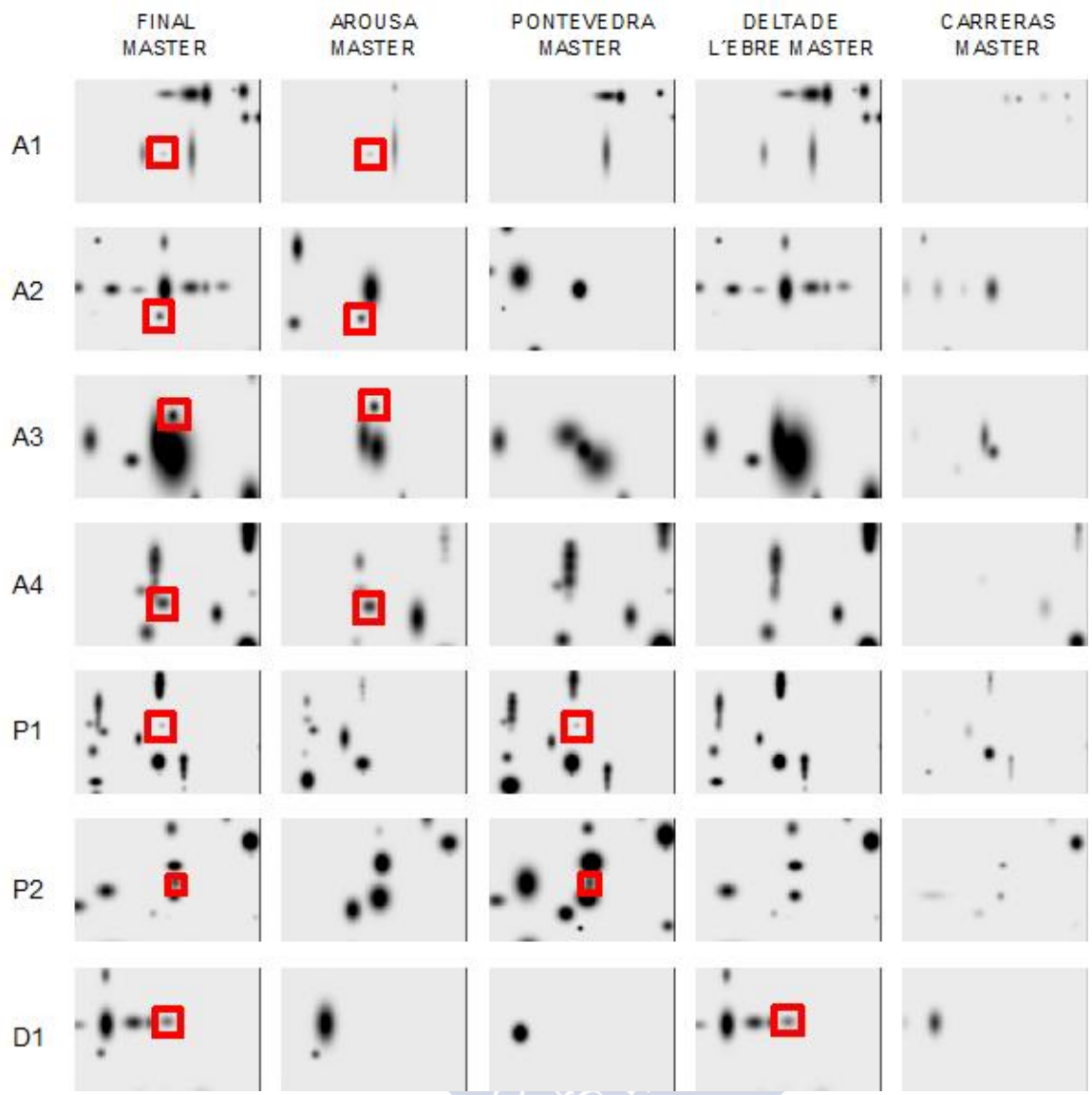

Fig. V.5. Fragments of master gels showing the spots exclusive of extracellular products of *Perkinsus olseni* from one region that were annotated. Left column corresponds to the areas of final master gel where the exclusive spots occur. The remaining columns correspond to the same areas in the master gel of each region. Each file corresponds to an exclusive spot labelled with the spot code (the same as in Table V.4). A- Ría de Arousa; P - Ría de Pontevedra; C – Carreras River; D - Delta de l´Ebre.

Table V.4. Peptides annotated after searching in Blast database against Alveolata group in non-redundant proteins database. The C before numbers correspond to spots common to all *Perkinsus olseni* regions, "A" corresponds to exclusive spots of Ría deArousa region, "P" to exclusive spots exclusive of Ría de Pontevedra, "D" to exclusive spots exclusive of Delta de l´Ebre and finally "H" exclusive spots of Carreras river region. Protein identification: show the protein name of identified peptides in the database. A.no.: is the accession number of proteins to database. Biological function shows the function of the protein according to Gene Ontholgy Biological Process. Sco: Displays a score to BLAST results; the higher score the better the match to the searched sequence.E-value: Expected value describes the number of hits expected to see by chance when searching a database of a particular size. Mw: Molecular weight. pI: Isoelectric point.

Spot code	pI obs/pI theor	Mw obs/Mw theo (kDa)	Identified protein; organism	A. no.	Biological function	Peptide sequence	Sco	E value
C1	6.2/5.35	49/44.61	Cathepsin b, putative; *Perkinsus marinus*	XP_002782575.1	Proteolysis	VAVYSPEEEAQHXA	47.1	0.040
C2	6.6/-	50/-	No identification	-	-	QGPCKPVT, NXXDSFTCR	-	-
C3	6.2/-	49/-	No identification	-	-	LAYDSTVR	-	-
C4	6.55/6.54	40/30.5	Signal recognition particle receptor subunit beta, putative; *Perkinsus marinus*	XP_002771937.1	Receptor	EAAESLY	24.8	11
C5	6.55/-	40/-	No identification	-	-	VTVSADS, RGEAEKFL	-	-
C6	6.5/-	28.2/-	No identification	-	-	AEAPESP, EGDMATLNY	-	-
C7	5.8/8.69	17.84/15.78	Hypothetical protein N-acetyl transferase superfamily; *Perkinsus marinus*	XP_002785377.1	N-Acetyltransferase activity	VVAMYLVVNP, EAAESLY	34.1	0.055
C8	5.8/-	7.84/-	No identification	-	-	AGVQNDAG, VVTVSLP	-	-
C9	5.4/6.54	14.74/30.46	Signal recognition particle receptor subunit beta, putative; *Perkinsus marinus*	XP_002771937.1	Receptor	EAAESLY	24.8	11

Spot code	pI obs/pI theor	Mw obs/Mw theo (kDa)	Identified protein; organism	A. no.	Biological function	Peptide sequence	Sco	E value
C10	6.6/4.56	12.1/7.92	Hypothetical uncharacterised protein *Perkinsus marinus*	XP_002788789.1	-	ASDLAHLHYDG	38	5E-04
A1	5.52/5.19	68.75/59.45	Heat shock protein 60; *Perkinsus marinus*	XP_002785716.1	Response to stress	LNDALNA, EDFDPALLGSC	35.8	0.004
A2	6.65/5.89	42/41.88	Phosphoserine aminotransferase; *Perkinsus marinus*	XP_002788117.1	L-Serine biosynthetic process	LAEDSNGFYNA, DSNGFYNAPV	32.9	0.37
A3	7.38/7.51	40/38.8	Pepsin A precursor; *Perkinsus marinus*	XP_002779904.1	Proteolysis	LGTLQVGD	23.5	30
A4	5.57/8.69	18.15/15.78	Hypothetical uncharaterised protein *Perkinsus marinus*	XP_002785377.1	-	VVAMYLVV	27.4	0.8
P1	5.95/6.28	18.8/17.00	Peroxiredoxin V; *Perkinsus chesapeaki*	ABV22156.1	Oxidation- reduction process	MXADXKADTA	27.4	2.1
P2	5.62/-	16.4/-	No identification	-	-	YDCGYTXDQGSGAP, AVCASDC	-	-
D1	6.9/-	45.5/-	No identification	-	-	ADEGXV, GEAEKFX, KDYPY	-	-

V.5. DISCUSSION

Results showed that *P. olseni* is able to release a high amount of proteins: up to 144 different spots were found in gels from ECPs of parasites from Delta de l'Ebre, which suggests a complex host-parasite biochemical interaction through the infection process. Furthermore, a remarkable variability in the protein profile of *P. olseni* ECPs depending on the geographic origin was found, because the percentage of similitude between clones within a region was higher than between clones of different regions. Interestingly, the variability of the protein profile of *P. olseni* ECPs is higher than that of cellular proteins recorded in chapter IV, because the percentage of similitude between clones within a region and between regions corresponding to ECPs was lower than those of cellular proteins, in spite of the number of cellular proteins was much higher than that of released proteins. Genetic and physiological variability depending on geographic origin had also been found in *P. marinus* (Reece et al., 1997, 2001; Thompson et al., 2011).

The comparison of *P. olseni* ECPs between regions showed that the two closest regions were Carreras River and Delta de l´Ebre followed by Ría de Pontevedra and Delta de l´Ebre, while Ría de Arousa was the most distant region, which is difficult to explain and lacks geographic proximity support. These results are not consistent with those based on either *P. olseni* cellular proteins (Chapter IV) or microsatellites of *P. olseni* genomic DNA from the same locations (Chapter III), which showed more similitude between *P. olseni* clones from the Atlantic coast locations with regard to the clones from the Mediterranean coast. As discussed in chapter IV, it is difficult to estimate the influence of the host species source of *P. olseni* clones on the variability of the protein profiles of ECPs; in this case, the host species source seemed to play a less important role for differences between *P. olseni* clones of different regions. The comparison between ECPs was based on much fewer proteins than that between cellular proteins, which confers a more solid base to the latter to infer similitude between *P. olseni* clones from different geographic origin.

Regarding protein annotation of sequences obtained from spots, the low success in the protein identification could be explained by the low quantity of ECP sequences of *P. olseni* or other species of the Alveolata group in the database, even after the complete genome of *P. marinus* has been sequenced (Joseph et al., 2010). Additionally, the low intensity (low protein concentration) of some spots made difficult obtaining a good spectrum by MS. Three annotated proteins corresponded to 4 spots shared by *P. olseni* ECPs from all the regions: One of these proteins, the signal recognition particle receptor subunit β (SRβ), corresponded to two different spots; why the same peptide sequence, annotated as SRβ, was identified in two spots with so different pI and molecular weight is puzzling. This receptor, only found in eukaryotes, is part of the complex signal recognition particle (SRP), a ribonucleoprotein complex that translocates proteins across the membrane to enter the secretory pathway (Egea

et al., 2008; Akopian et al., 2013; Nyathi et al., 2013). This complex consists of three GTPases: the SRP54 subunit of the SRP and the α- and β- of the SR receptor (Helmers et al., 2003). The presence of SRβ in the ECP suggests that *P. olseni* could use the SRP complex as a transport channel to release proteins. Once the protein crosses into the complex, the SRβ could be released together with the protein. Nevertheless, the precise timing and coordination of events during the signal sequence transfer still remain poorly understood (Helmers et al., 2003; Nyathi et al., 2013). Other protein shared by *P. olseni* ECPs from all the regions was a cysteine peptidase, cathepsin B. Peptidases are essential for the establishment and survival of the parasites; they play an important role in the development and pathogenesis of several parasitic infections and have been proposed as targets in the structure-based strategy of drug design (Sakanari et al., 1997; Schaeffer et al., 2011). Cysteine peptidases from trophozoites of *Entamoeba histolytica* were found to have a cytopathic effect on mammalian cells (Keene et al., 1990); cysteine proteases of *Plasmodium falciparum* are involved in the degradation of host haemoglobin (Rosenthal, 1995); cathepsin B has been purified from intracellular and extracellular extracts from all stages of *Trypanosoma cruzi* (Nóbrega et al., 1998); cysteine peptidase involvement in host cell invasion by sporozoites of *Eimeria tenella* was deduced because cell invasion was inhibited by cysteine peptidase inhibitors (Schaeffer et al., 2011). In the case of the genus *Perkinsus*, other type of proteases, serine peptidases, have been found in *P. marinus* ECPs and been proposed as possible virulence factors responsible for tissue degradation in infected oysters (La Peyre, 1995b, 1996); the major extracellular protease (a N-glycosylated serine peptidase) produced by *P. marinus in vitro* was characterised and designed as perkinsin (Faisal et al., 1999). More recently, several protease sequences (cathepsin-like cysteine protease, subtilisin-like serine protease, rhomboid-like protease 1, cysteine protease, ATP-dependent protease, serine protease, metacaspase 1 precursor, and ubiquitin-specific proteases) were also identified in the *P. marinus* genome (Joseph et al., 2010) and would be expected to degrade host protein substrates to acquire nutrients necessary for normal cell function and proliferation (Soudant et al., 2013). No protease activity had been found in previous studies on *P. olseni* ECPs although other hydrolytic enzyme activities were reported (Casas et al., 2002b, 2008, 2009). Hosts counteract the proteases released by parasites by producing protease inhibitors. Serine protease inhibitors that may inhibit *P. marinus* proliferation were found in *C. virginica* oysters (Faisal et al., 1998; Oliver et al., 2000; Xue et al., 2006, 2009; La Peyre et al., 2010). The occurrence of cysteine peptidase in *P. olseni* ECPs raise the question of the production of protease inhibitors in the Manila clam *R. philippinarum*; 23 contigs with homology to serine, cystein, Kunitz-type and Kazal-type protease inhibitors and metalloprotease inhibitors were identified in Manila clams (Moreira et al., 2012). Other identified protein shared by *P. olseni* ECPs from all the regions was a hypothetical protein of the N-acetyl transferase superfamily; there are many different proteins within this superfamily, these enzymes

use acetyl coenzyme A to transfer an acetyl group to a substrate, a reaction implicated in various functions of cellular processes.

Three annotated proteins -pepsin A precursor, phosphoserine aminotransferase, and heat shock protein (HSP) 60- corresponded to spots exclusively found in the ECPs of the four *P. olseni* clones from Ría de Arousa. Pepsin A precursor is an aspartic protease occurring in many organisms. The catalytic site is formed by two aspartate residues, one of which have to be protonated and the other deprotonated for the activation of the protein (Campos and Sancho, 2003). This is the second protease identified in *P. olseni* ECPs in this study. Considering the important role of proteases released by parasites in their pathogenicity, the fact that the spot corresponding to pepsin A precursor was not found in clones from the other regions could support higher virulence of *P. olseni* clones from Ría de Arousa. Phosphoserine aminotransferase (PSAT) is involved in serine biosynthesis; L-serine serves as a building block for protein synthesis and also plays an important role in various metabolic pathways for the generation of essential compounds, including glycine, L-cysteine, L-methionine, phosphatidyl-L-serine, sphingolipids, taurine, porphyrins, purines, thymidine, and neuromodulators D-serine that are essential for the growth of the organisms (Ali and Nozaki, 2006). HSP 60 is included in a highly conserved family of proteins present in all organisms; they were originally identified as cellular proteins produced in response to stress or elevated temperature, but most HSPs were found to be constitutively expressed in cells and they are essential for cellular growth under normal conditions (Syin and Goldman, 1996). Most of these proteins play essential roles in protein biosynthesis and are involved in the transport, translocation and folding of proteins. Extracellular HSPs are important mediators of intercellular signaling and transport, once released may then bind to the surfaces of adjacent cells and initiate signal transduction cascades as well as the transport of cargo molecules (Calderwood et al., 2007). The role of extracellular HSP 60 is not well understood, induction of immunosupression was suggested (Flohe et al., 2007) and they have been shown to induce inflammation (Tian et al., 2013) or even autoimmune aggression (Cappello et al., 2009); tumour cells have been shown to release HSP 60 (Merendino et al., 2010). Thus, pathogenic effect of HSP60 released from *P. olseni* could be suspected; if so, it would further support higher virulence of *P. olseni* clones from Ría de Arousa.

Peroxiredoxin V annotation corresponded to one spot occurrying exclusively in the four *P. olseni* clones from Ría de Pontevedra. Peroxiredoxins belong to the peroxidase family. In some parasites they may participate in the defence against the host attacks involving reactive oxygen intermediates (ROIs), because peroxiredoxins prevent the accumulation of H_2O_2, and even exert potent immune-modulatory effects (Kawazu et al., 2008; Ishii et al., 2012; Robinson et al., 2013). Peroxiredoxin V was reported within *in vitro* cultured *P. chesapeaki and P. olseni* cells, the latter also deriving from Ría de Pontevedra (Chapter VI), and the gene coding this protein was

identified in the genome of *P. marinus* (Joseph et al., 2010). *P. marinus* is able to inhibit the host production of reactive oxygen intermediates (ROIs) (Anderson, 1999; Volety and Chu, 1995) and to resist them (Schott et al., 2003); similarly, *P. olseni* has to face ROIs released by encapsulating haemocytes (Soudant et al., 2013); peroxiredoxin V could be involved in the *Perkinsus* spp. mechanisms of neutralising ROIs produced by hosts. Accordingly, *P.olseni* clones from Ría de Pontevedra could be more resistant to host attack than those from the other regions.

In conclusion, relative higher variability has been found in the protein profiling of ECPs of *P. olseni* among regions of Spanish coasts than that previously reported in cells. Two types of proteases have been detected in *P. olseni* ECPs, while previous studies had not been found any protease activity in ECPs of this species. Differences found between ECPs of *P. olseni* clones from different regions suggest differences in virulence or in resistance to host attack that should be further assessed.

VI. COMPARISON OF PROTEIN EXPRESSION PROFILES BETWEEN THREE *Perkinsus* spp.: *P. olseni*, *P. marinus* and *P. chesapeaki*

- **The content of this chapter has been published in**:

Fernández-Boo, S., Chicano-Gálvez, E., Alhama, J., Barea, J. L., Villalba, A., Cao, A., 2014. Comparison of protein expression profiles between three *Perkinsus* spp., protozoan parasites of molluscs, through 2D electrophoresis and mass spectrometry. J.Invertebr. Pathol. 118, 47-58.

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VI.1. ABSTRACT

The genus *Perkinsus* includes protozoan parasites of a wide range of marine molluscs worldwide, some of which have been responsible for heavy mollusc mortalities and dramatic economic losses. This study was performed with the aim of increasing the knowledge of *Perkinsus* spp. proteome. Proteins extracted from *in vitro* cultured cells of three species of this genus, *P. marinus*, *P. olseni* and *P. chesapeaki*, were analysed using 2D electrophoresis. Four gels from each species were produced. Qualitative and quantitative comparisons among gels were performed with Proteamweaver software. Cluster analysis grouped the four gels of each *Perkinsus* sp.; furthermore, *P. marinus* and *P. olseni* gels were grouped in a cluster different from *P. chesapeaki*. Around 2000 spots of each species were considered, from which 213 spots were common to the 3 species; *P. chesapeaki* and *P. marinus* shared 310 spots, *P. chesapeaki* and *P. olseni* shared 315 spots and *P. marinus* and *P. olseni* shared 242 spots. A number of spots were exclusive of each *Perkinsus* species: 1161 spots were exclusive of *P. chesapeaki*, 1124 of *P. olseni* and 895 of *P. marinus*. A total of 84 spots, including common and species-specific ones, were excised from the gels and analysed using MALDI-TOF and nESI-IT (MS/MS) techniques. Forty two spots were successfully sequenced, from which 28 were annotated, most of them clustered into electron transport, oxidative stress and detoxification, protein synthesis, carbohydrate metabolism, signal transduction, metabolic process and proteolysis.

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VI.2. INTRODUCTION

Parasites of the genus *Perkinsus* cause mortality of marine molluscs worldwide (Villalba et al., 2004). The International Society of Protistologists, in its classification of protists (Adl et al., 2012), included the genus *Perkinsus* in the group Perkinsidae, which is grouped with dinoflagellates and other protists in the Protalveolata, within the first rank group Alveolata, in the supergroup SAR (Stramenophiles, Alveolata, Rhizaria). Ten species of *Perkinsus* have been described, although only seven are considered valid (Villalba et al., 2011): *Perkinsus marinus* (Mackin et al., 1950)*, Perkinsus olseni* (Lester and Davis, 1981)*, Perkinsus qugwadi* (Blackbourn et al., 1998), *Perkinsus chesapeaki* (McLaughlin and Faisal, 2000), *Perkinsus mediterraneus* (Casas et al., 2004), *Perkinsus honshuensis* (Dungan and Reece, 2006) and *Perkinsus beihahiensis* (Moss et al., 2008). The World Organisation for Animal Health (OIE) has included *P. marinus* and *P. olseni* in the list of notifiable diseases (http://www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2012/). *P. marinus* is responsible for heavy mortalities of eastern oyster *Crassostrea virginica* populations along the Atlantic coast of North America from Maine (USA) (Ford, 1996) to the Yucatan Peninsula (Mexico) (Gullian-Klanian et al., 2008). It has been further detected in oysters *Crassostrea gigas* (Calvo et al., 1999) and *Crassostrea ariakensis* (Calvo et al., 2001) and clams *Mya arenaria*, *Macoma balthica*, *Macoma mitchelli* (Coss et al., 2001) and *Mercenaria mercenaria* (Pecher et al., 2008) from the Atlantic coast of USA and has been reported infecting *Crassostrea corteziensis* and *Saccostrea palmula* in the Pacific coast of Mexico (Cáceres-Martínez et al., 2008; Cáceres-Martínez et al., 2012) and *Crassostrea rhizophorae* in Brazil (da Silva et al., 2013). *P. marinus* geographic and host ranges overlap those of *P. chesapeaki*. The latter was first described infecting the softshell clam *Mya arenaria* in the Chesapeake Bay (USA). *Perkinsus andrewsi*, which was described infecting the clam *Macoma balthica* from the same bay (Coss et al., 2001), was considered synonym of *P. chesapeaki* (Burreson et al., 2005). Since then this parasite has been detected in clams *M. mitchelli*, *M. mercenaria*, *Tagellus plebeius*, *Cyrtopleura costata*, *Rangia cuneata*, *Mulinia lateralis* and the oyster *C. virginica* (Reece et al., 2008; Villalba et al., 2011). *P. marinus* almost exclusively infects *C. virginica* whereas *P. chesapeaki* predominantly infects clams in the shared geographic range (Reece et al., 2008). It seems than clams are not the optimal host for *P. marinus* and their infections could be incidental (Pecher et al., 2008). Recently, *P. chesapeaki* has been detected in clams *Ruditapes decussatus* from France (Arzul et al., 2012) and *Ruditapes philippinarum* from Spain (Ramilo et al., 2012). This parasite has been associated with declines in commercial clam landings (Dungan et al., 2002; McLaughlin and Faisal, 2000). *P. olseni* was first described in the blacklip abalone *Haliotis ruber* from Australia (Lester and Davis, 1981); *Perkinsus atlanticus*, which was described infecting clams *Ruditapes decussatus* from Portugal (Azevedo, 1989), was considered a synonym of *P. olseni* (Murrell et al., 2002). *P. olseni* infects a wide range of mollusc species in coastal waters of Asian, Australasian,

European and South American countries (Casas et al., 2002a; Cremonte et al., 2005; Choi and Park, 2010; Dungan et al., 2007; Goggin and Lester, 1995; Hamaguchi et al., 1998; Leethochavalit et al., 2004; Park et al., 2006; Sanil et al., 2010; Wu et al., 2011). *P. olseni* has been blamed for mass mortalities of *R. philippinarum* in Korea (Park and Choi, 2001) and China (Liang et al., 2001) and has been associated with mortality of *R. decussatus* in Portugal (Azevedo, 1989) and Spain (Villalba et al., 2005). The life cycle of *Perkinsus* spp. involves that a cell type, usually called trophozoite, proliferates throughout host tissues undergoing successive bipartitioning to yield daughter cells that stay together inside a wall; daughter cells become independent and mature into trophozoites. When host infected tissues are incubated in fluid thioglycollate medium (which mimics natural tissue rotting after host death), the trophozoites transform into a resistant stage, called hypnospore. When hypnospores are transferred into seawater, zoosporulation begins and progresses with successive karyokinesis and cytokinesis, leading to formation and release of hundreds motile biflagellated zoospores. Zoosporulation is rarely seen in *P. marinus* when placed in sea water (da Silva et al., 2013; La Peyre, pers. comm.). Trophozoites, hypnospores and zoospores are able to initiate infection in a healthy host (Villalba et al., 2004). Procedures for *in vitro* cultivation of *Perkinsus* spp. have been established (La Peyre, 1996).

Various approaches have been used in biological, ecological and evolutionary studies to characterise and understand the phenotypic variability at the biochemical level, such as genomics, transcriptomics and proteomics (Biron et al., 2006; Derome and Bernatchez, 2006; Roberge et al., 2008). All of them have limitations and numerous studies have shown examples of lack of correlation between mRNA and protein abundance (Griffin et al., 2002; Gygi et al., 1999; Ideker et al., 2001; Lippolis and Reinhardt, 2010). This lack of correspondence has been used as a justification for the application of proteomics in studying expression differences between species or strains (Brobey et al., 2006; Chan et al., 2005; Enard et al., 2002; Wang et al., 2008), with the aim of the identification of putative virulence factors as it was shown in several proteomic studies on parasites of the Alveolata group (Briolant et al., 2010; Cuervo et al., 2007; Drummelsmith et al., 2003). Information derived from these techniques are complementary, thus the combination of all, including metabolomics, are necessary for an in-depth understanding of parasite biology, accelerate the search for new proteins and therefore facilitate a comprehensive view of the cellular process including gene expression, mRNA content, protein expression and cellular response.

The purpose of our study was to establish 2-DE as a tool to study protein expression patterns of the 3 *Perkinsus* spp. that have been blamed for host mortality, *P. marinus*, *P. olseni*, and *P. chesapeaki*, with the ultimate goal of employing proteomic technologies to identify common and species-exclusive proteins. If differences were found, they could contribute to a better understanding of *Perkinsus* spp. infection mechanisms, and differences of virulence and host preferences among species. Various proteins related with detoxification, electron transport, synthesis and signal

transduction have been identified. The functions of identified proteins have been assigned according to Gene Ontology and the potential pathways of these proteins are discussed in light of bioinformatics analyses.

VI.3. MATERIALS AND METHODS

VI.3.1. In vitro *clonal cultures*

Isolates from *P. olseni* (Pag6-07-P2-A1), *P. marinus* (GTLA-34) and *P. chesapeaki* (PRA-65) were used to develop *in vitro* clonal cultures. *P. olseni* culture was established from gills of clams *R. decussatus* from the Ría de Pontevedra (Galicia, NW Spain), following the method of La Peyre et al. (1993); *P. marinus* culture was provided by Jerome La Peyre (Department of Veterinary Science, Louisiana State University Agricultural Center), which had been established from heart from an infected *Crassostrea virginica* from Lower Barataria Bay (Louisiana, USA); *P. chesapeaki* culture was provided by Chris Dungan (Maryland Department of natural Resources, Cooperative Oxford Laboratory), which had been established from hypnospores of *Mya arenaria* from Chesapeake Bay (Maryland, USA). All cultures were maintained at 25ºC and were subcultured every 2-3 months in the protein-free culture medium JL-ODRP-2A (Casas et al., 2002b). *In vitro* clonal cultures of *P. olseni*, *P. marinus* and *P. chesapeaki* were established as described Casas and La Peyre (2009). Parasites in exponent phase of growth were collected by centrifugation at 1000g for 10 min at 25°C. Parasite density was estimated by counting with a haemocytometer.

VI.3.2. Protein extraction

Proteins were extracted by suspending $150x10^6$ trophozoites from *in vitro* clonal cultures (one culture of each parasite species) in lysis buffer (8M urea, 2M thiourea, 2% CHAPS, 1% DTT, 0.8% ampholites (pH 3-10) and a 1/100 dilution of a protease inhibitor (Sigma Protease Inhibitor P2714). Proteins were solubilised for 2 h 30 min at 4°C with vigorous shaking. The lysate was centrifuged at 16000g for 30 min at 4ºC. The supernatant was purified using the 2-D Clean Up Kit (GE Healthcare) and finally, the pellet was resuspended in rehydration solution (8M urea, 2% CHAPS, 0.5% IPG buffer, 0.8% ampholites (pH 3-10), 40mM DTT and bromophenol blue traces). Protein concentration was measured using the DC protein assay kit (Bio-Rad), according to Lowry et al. (1951).

VI.3.3. Two dimensional electrophoresis (2DE)

For the first dimension separation, aliquots of 150 µg of protein samples were diluted to a final volume of 350 µl in rehydration solution and were incubated for 30 min at room temperature (23-26°C). Samples were centrifuged 1 min at maximum

speed to remove bubbles and loaded onto the immobilized pH gradient (IPG)-strips (18 cm, pH 4-7 linear, GE Healthcare) by in-gel rehydration. After 6-h passive and 6-h active (50V) rehydration, Iso-Electro Focusing (IEF) was performed (20ºC, 50 µA/strip) in a Protean®IEF System (Bio Rad) using six steps: 500 V, 90 min; 1000 V, 90 min; 2000 V, 90 min; 4000 V, 90 min; 8000 V, 120 min, and 8000 V, until 60 000 Vh (4 h) according to Romero-Ruiz et al. (2006). After IEF, proteins were reduced (10 mg/ml DTT, 20 min) and alkylated (45 mg/ml iodoacetamide, 20 min) in equilibration buffer (6M urea, 50mM Tris pH 8.8, 2% SDS, 30% glycerol) before separation in the second dimension. The proteins on the equilibrated IPG strips were separated across 12.5% SDS-PAGE gels using a vertical system (PROTEAN Plus Dodeca Cell, BioRad) and standard Tris/glycine/SDS buffer. Gels were run at 2.5 W/gel for 15 min followed by 12.5 W/gel for 5h 30 min. Molecular weight markers (BioRad) were run in the second dimension next to the problem sample for protein size determination.

VI.3.4. Protein visualisation and image analysis

Four replicates gels from the same protein extraction of each species were prepared. Protein spots in the gels were visualized by Sypro ruby (BioRad) staining following the manufacturer's specifications. Images of the four replicate gels from each *in vitro* clonal culture, (4 replicates x 3 species = 12 gels) were digitised with a FXImager scanner (BioRad). Automatic gel analysis using the Proteomweaver™ software 4.0 (Bio-Rad) was performed to properly assign the spots, correcting possible slight better match spots correcting possible slight gel deformations. All the images were highly reproducible in terms of number of spots, position and intensity. Spot patterns were analysed and compared between parasites in search for differences in protein expression profiles. The gels were calibrated using a select set of reliable identification landmarks distributed throughout the entire gel to determine experimental isoelectric point (pI) and molecular weight (Mw) coordinates for each single spot. All spot volumes were normalized to get normalized spot intensities. The theoretical Mw and pI values were obtained with the compute pI/Mw tool at the ExPASy Proteomics Server (http://www.expasy.org/tools/pi_tool.html).

VI.3.5. Protein identification and database search

Fourteen spots that occur in the gels of the three *Perkinsus* spp. at the same position (common spots), 14 spots that were exclusive of *P. marinus*, 14 spots exclusive of *P. olseni* and 14 spots exclusive of *P. chesapeaki* were selected for protein identification. The number of spots selected for identification was limited by funding availability, thus the selection criteria was to choose the most intense spots among the common and the exclusive ones. Candidate protein spots were excised from the gels with a robot station (Genomic Solutions Propic®) at the Proteomics Facilities of

University of Córdoba (SCAI, University of Córdoba, a member of ProteoRed network). In the case of the 14 spots common to the three *Perkinsus* spp., one spot from a gel of each species was excised (14 x 3 species = 42), to assure that the proteins were really the same in the three species. A total of 84 (42 exclusive spots + 42 common spots) excised spots were sent to Proteomics Laboratory of Autonomous University of Barcelona (LP-CSIC/UAB) and digested with trypsin as described by Romero-Ruiz et al. (2006). Extracted peptides were analysed by matrix-assisted laser desorption/ ionisation/time-of-flight mass spectrometry (MALDI-TOF MS) using a Voyager DE-PRO instrument (Applied Biosystems, Foster City, CA, USA). An ion-trap mass spectrometer Finningan LCQ IT (ThermoQuest, Finnigan MAT, San José, CA, USA) was used to verify proper digestion and sequencing by nanoelectrospray ion trap MS/MS (nESI-IT MS/MS).

Peaks software (Thermo Electron Corporation, San Jose, CA USA) was used for *de novo* peptide sequencing. SEQUEST software was used for automated searching protein sequences against Uniprot - SwissProt database, in first time sequences were searched against non-redundant protein database of all organisms and then against Alveolate taxa database. All sequence tags obtained were manually confirmed and were submitted to a homology search using the pBLAST algorithm software (NCBI, USA). Data were contrasted against Alveolata taxa included in NCBI (National Center for Biotechnology Information, Maryland, USA) database. Correct identifications were considered valid with a score (ALC%) ≥ 65 (PEAKS) and a Sf value ≥ 0.90 (SEQUEST).

VI.3.6. Statistical analysis

Protein expression similarity between *Perkinsus* spp. was evaluated by considering only the spots occurring in at least one species, with a low variation coefficient among the four gels of the same species, to assure homogeneous intensity, an average linkage cluster analyses of the 12 gels (4 gels x 3 *Perkinsus* spp.) was performed based on the intensity of the spots to produce a dendrogram with correlation coefficient distance. Additionally, differences between the three *Perkinsus* spp. in the intensity of the 14 common spots that had been excised for protein identification were tested through one way ANOVA, followed by Tukey´s multiple comparison tests. MINITAB 15 software was used for the statistical analysis.

VI.4. RESULTS

Fig. VI.1 shows representative 2-DE gels of *P. olseni*, *P. marinus* and *P. chesapeaki*. An average of 1894 spots (range: 1518 – 2093) were detected in the gels of *P. olseni*, 1660 (range: 1394 – 2101) in those of *P. marinus* and 1999 (range: 1881 – 2125) in those of *P. chesapeaki.* The comparison between the three *Perkinsus* spp. showed 213 spots shared by the three species; *P. chesapeaki* and *P. marinus* shared

310 spots, *P. chesapeaki* and *P. olseni* shared 315 spots and *P. marinus* and *P. olseni* shared 242 spots. A number of spots were exclusive of each *Perkinsus* species: 1161 spots were exclusive of *P. chesapeaki*, 1124 of *P. olseni* and 895 of *P. marinus* (Fig.VI.2). Cluster analysis grouped the four gels of each *Perkinsus* sp.; furthermore, *P. marinus* and *P. olseni* gels were grouped in a cluster different from *P. chesapeaki*, although the distance between the *P. chesapeaki* cluster and the *P. marinus* + *P. olseni* cluster was short (Fig. VI.3). Significant differences between the three *Perkinsus* spp. in the intensity of some of the 14 common spots that were excised for identification were found (Fig. VI.4).

Table VI.1 summarises the data of the identified spots. Forty-two of the 84 excised spots were successfully sequenced and 28 were identified, which represents 67% of successfully sequenced spots. Regarding common spots, C3 and C7 were identified as receptor for activated C kinase and pseudouridine synthase, respectively, by the same peptide sequences in all three species; spot C1 had peptides identified pairwise among the three species, corresponding to vacuolar ATPase b subunit; spot C13 was identified as phosphate acetyltransferase in *P. chesapeaki* and *P. marinus*; and spot C14 was identified as vacuolar ATPase subunit g in *P. olseni* and *P. marinus*. The spot C2 was identified as malate dehydrogenase only in *P. olseni*. Five more common spots were sequenced but they could not be annotated; their sequences showed some differences between the 3 *Perkinsus* spp. except for one of them, which showed the same sequence in the three *Perkinsus* spp. With regard to species-exclusive spots, six spots were identified in *P. olseni*: O1 as an uncharacterized protein of *P. marinus*; O3 as formate dehydrogenase; O6 as a proteasome subunit; O7 as triose phosphate isomerase and O14 as peroxiredoxin V. Two specific spots were successfully identified in *P. marinus*: M1 as phosphoglycerate kinase and M4 as 40S ribosomal protein S3. Seven specific spots were identified in *P. chesapeaki*: three of them corresponding to spots Ch1, 6 and 9 were annotated as uncharacterized proteins of *P. marinus*; Ch7 as glutathione S-transferase; Ch8 as peroxiredoxin II; and Ch11 and Ch12 as peroxiredoxin V. According to Gene Onthology (GO) annotation of biological process, the identified proteins of three *Perkinsus* species could be classified in 7 categories (Fig. VI.5). The majority of the identified proteins were clustered into electron transport; other identified proteins were involved in antioxidant functions, protein synthesis, carbohydrate metabolism and signal transduction; a minority were involved in metabolic processes and proteolysis.

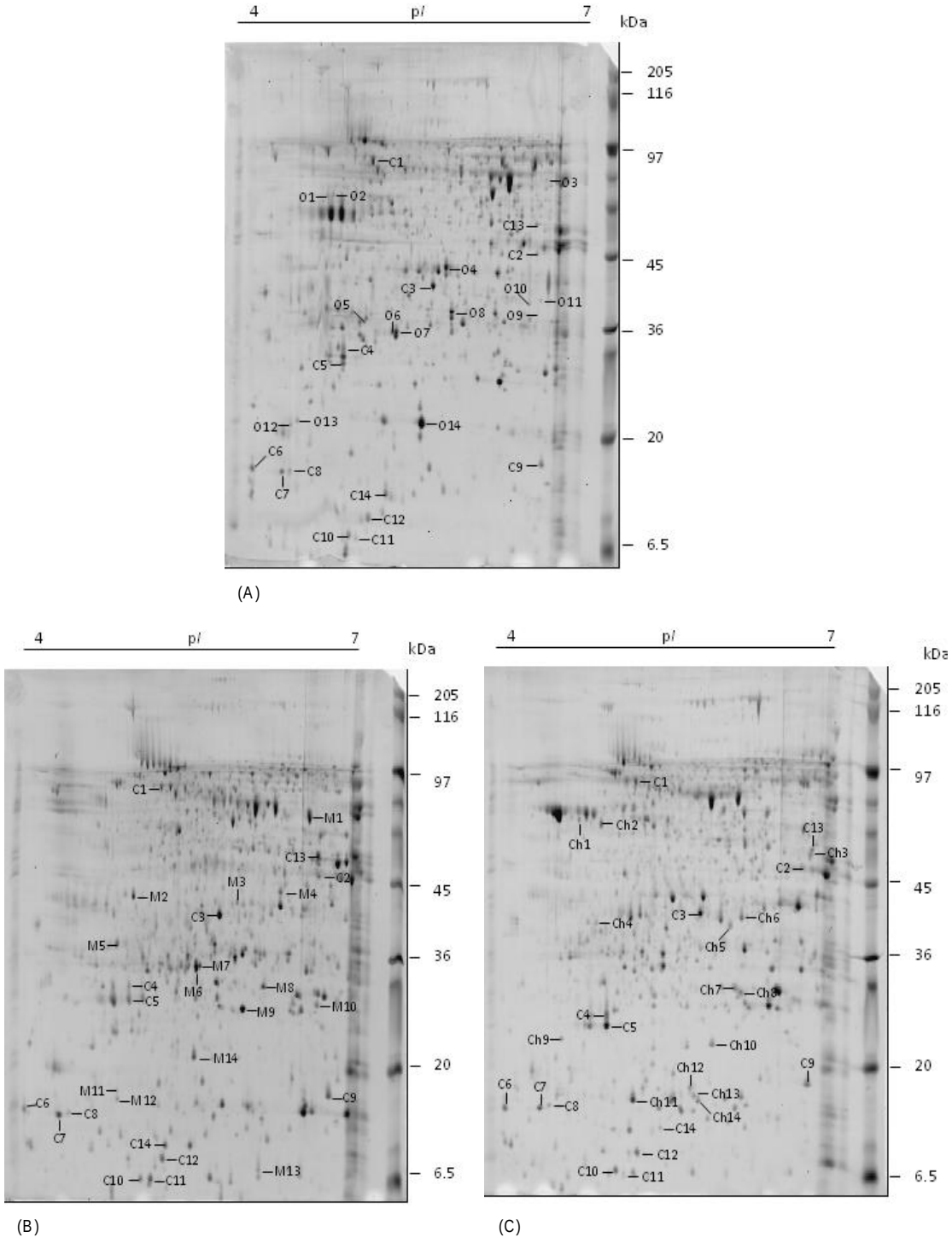

Fig. VI.1: Digitised images of Sypro ruby-stained gels produced by 2D-SDS-PAGE of *Perkinsus olseni* (A), *Perkinsus marinus* (B), and *Perkinsus chesapeaki* (C). The spots that were excised for identification are pointed out; the spots C1-14 were common to the three *Perkinsus* spp., O1-14 were exclusive of *P. olseni*, M1-14 were exclusive of *P. marinus*, and Ch1-14 were exclusive of *P. chesapeaki*.

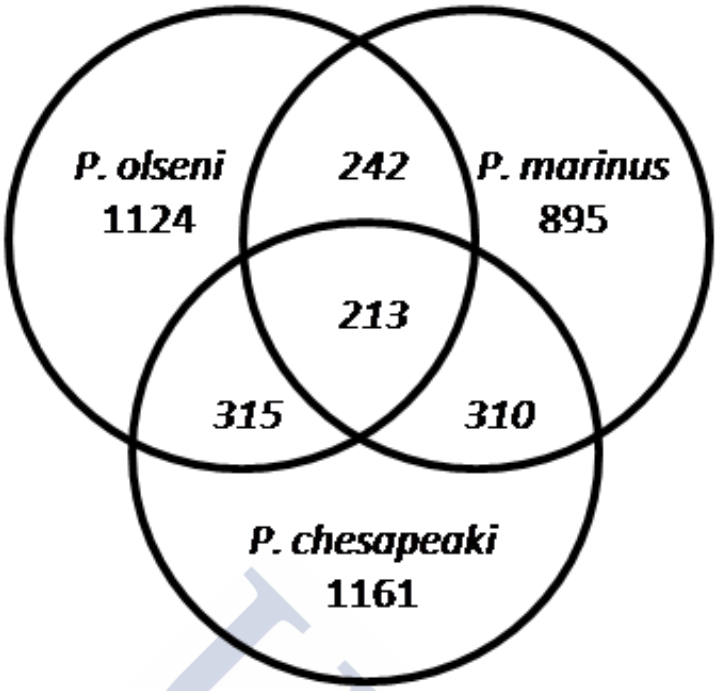

Fig. VI.2. Venn diagram showing the results of the protein analysis.

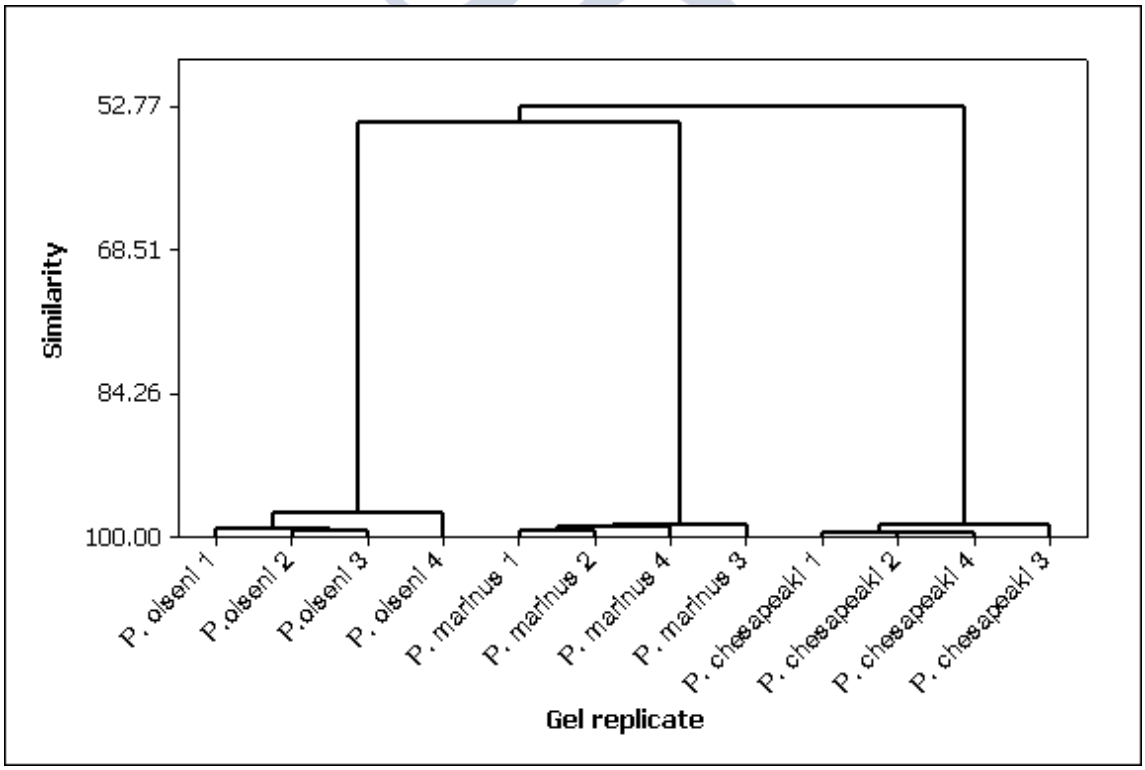

Fig. VI.3. Dendrogram of combined expression data corresponding to 4 gels of each *Perkinsus* spp. (*P. olseni*, *P marinus*, and *P. chesapeaki*) considering only the spots occurring in at least one species with a low variation coefficient among the four gels of the same species to assure homogeneous intensity. The 4 gel replicates of each species are technical replicates of the same sample.

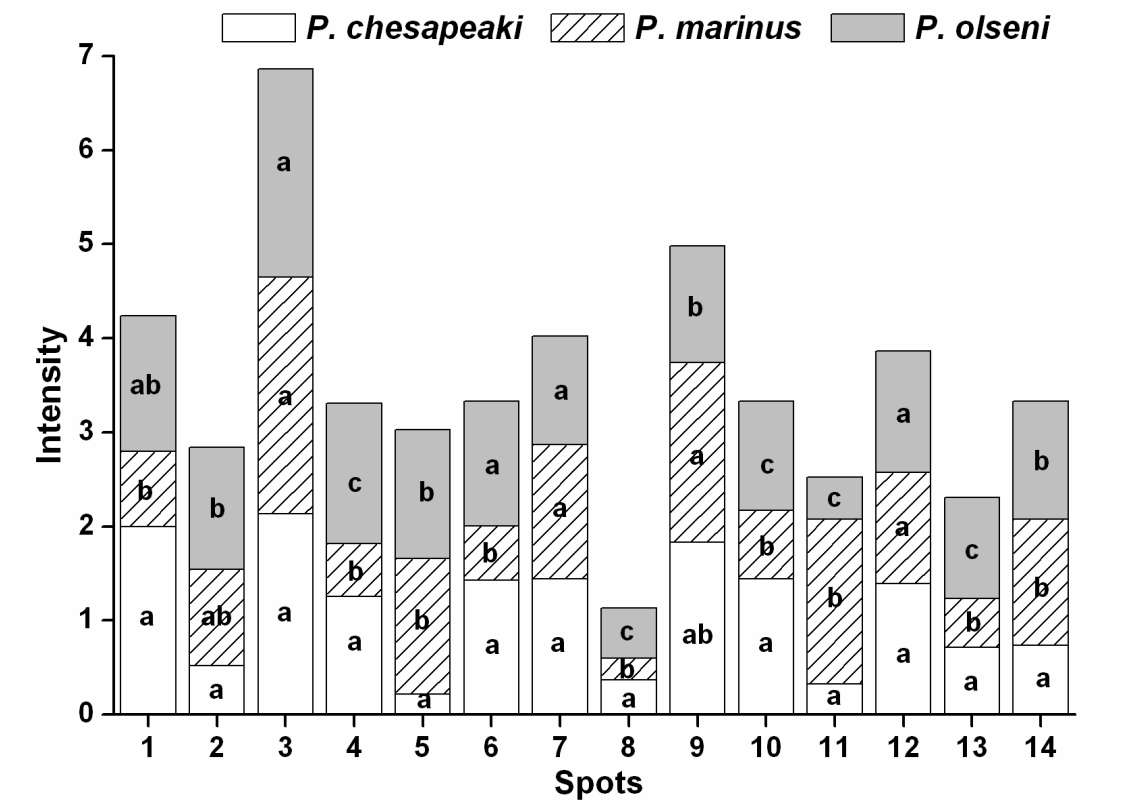

Fig. VI.4. Mean intensity corresponding to the 14 spots occurring in the gels of the three *Perkinsus* spp. that were excised for identification. *Perkinsus* spp. are distinguished with different bar patterns. Different letters in the patterns of each spot indicate significant differences between *Perkinsus* spp. Spot 1: vacuolar ATPase subunit b; spot 2: malate dehydrogenase; spot 3: receptor for activated C kinase, spot 7: pseudouridine synthase; spot 13: phosphate acetyl transferase; spot 14: vacuolar ATPase subunit g; the remaining spots were sequenced but did not match with proteins in databases.

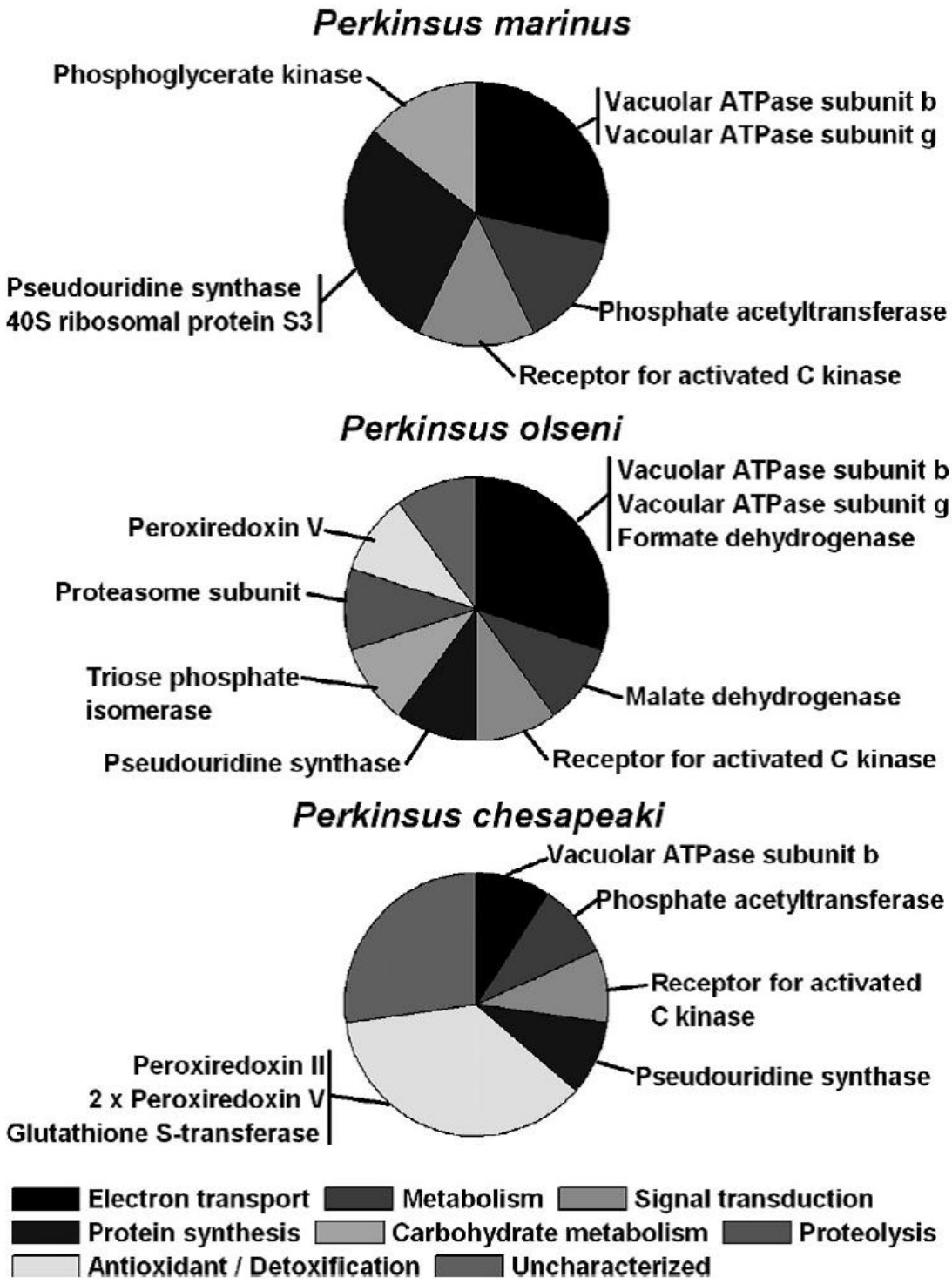

Fig. VI.5. Pie diagrams of each species showing the percentage of each protein group and the identified proteins in the study

Table VI.1. Peptides annotated after searching in Blast database against Alveolata group in non-redundant proteins database. The initial letter "C" in the code corresponds to spots common to the three *Perkinsus* spp., "Ch" corresponds to spots exclusively found in *P. chesapeaki*, "M" to spots exclusive of *P. marinus* and "O" to spots exclusive of *P. olseni*. Mw: Molecular weight. pI: Isoelectric point. Sf: The Sf score for each peptide is calculated by a neural network algorithm that incorporates the Xcorr, DeltaCn, Sp, RSp, peptide mass, charge state, and the number of matched peptides for the search; the higher the value of the Sf score, the better the peptide match. E-value: Expectation value. The number of different alignents with scores equivalent to or better than S that are expected to occur in a database search by chance. The lower the E value, the more significant is the score. Function was stated according to Gene Onthology.

Spot No.	Mw Theor./ Mw Exp.	pI Theor./ pI Exp.	PEAKS sequence	SEQUEST sequence	Sf	Identified protein; organism (automated search)	UNIPROT AC	Blast (manual search)	E-value	Function
C1^{o}	55.6 (94.0)	5.1 (5.2)		K.EM*IQTGISAIDTM*NSVVR.G	0.98	Vacuolar ATPase beta subunit; *Amphidinium carterae*	B4ZFY9		1,0E-10	
				K.LPLFSAAGLPHNEIAAQVCR.Q	0.92	Vacuolar ATPase beta subunit; *Amphidinium carterae*	B4ZFY9		3,0E-15	
				R.NDFEENGSM*ENVVLFM*NLANDPTIER.I	0.97	Vacuolar ATPase beta subunit; *Amphidinium carterae*	B4ZFY9		3,0E-20	
			FTAAVNAAAVTR			Tipc, putative; *Toxoplasma gondii*	B9QHT5		5,1E-03	
			MDLTAAEYFAYER			Putative vacuolar H^{+} ATPase subunit B; *Toxoplasma gondii*	Q86N78		2,0E-04	
C1^{m}				K.QVYPPINVLPSLSR.L	0.93	Chromosome undetermined scaffold_14; *Paramecium tetraurelia*	A0C0D1	Vacuolar ATPase beta subunit	2,0E-09	Electron Transport
			FMAAALNAAAVTR			Vacuolar ATPase beta subunit; *Amphidinium carterae*	B4ZFY9		8,0E-04	
				K.EM*IQTGISAIDTM*NSVVR.G	0.96	Vacuolar ATPase beta subunit; *Amphidinium carterae*	B4ZFY9		1,0E-10	
			GDAAVNAAAVTR			Putative uncharacterized protein; *Toxoplasma gondii*	Q1JSG6		5,1E-03	
			FDAAALNAAAVTR			Putative uncharacterized protein; *Toxoplasma gondii*	B9Q850		9,0E-03	
C1^{ch}			M(CamC)PETGLSALDTFNSVVR			Vacuolar ATPase beta subunit; *Amphidinium carterae*	B4ZFY9		2,0E-04	
			QVYPPLNVLPAESR			Chromosome undetermined scaffold_42; *Paramecium tetraurelia*	Q3SEB3		4,0E-03	
			FDAAALNAAAVTR			Putative uncharacterized protein; *Toxoplasma gondii*	B9Q850		9,0E-03	

Spot No.	Mw Theor./ Mw Exp.	pI Theor./ pI Exp.	PEAKS sequence	SEQUEST sequence	Sf	Identified protein; organism (automated search)	UNIPROT AC	Blast (manual search)	E-value	Function
C2^{o}	34.0 (46.0)	7.3 (6.7)	LVGVTTLDSVR			Malate dehydrogenase; *Heterocapsa triquetra*	Q5ENS5	Malate dehydrogenase	2,0E-03	Metabolism
C3^{o}	34.4 (40.0)	5.6 (4.9)	NVLEELTPPAEYK			Putative uncharacterized protein; *Theileria annulata*	Q4UAM3	Receptor for activated C kinase	2,0E-05	Signal traduction
			KPLEELTPPAEYK			Putative uncharacterized protein; *Plasmodium knowlesi*	B3L1K0		3,0E-03	
C3^{m}			NVLEELTPPAEYK			Putative uncharacterized protein; *Theileria annulata*	Q4UAM3		2,0E-05	
C3^{ch}			NVLEELTPPAEYK			Putative uncharacterized protein; *Theileria annulata*	Q4UAM3		2,0E-05	
			QPLEELTPAPEYK			Putative uncharacterized protein; *Babesia bovis*	A7AT99		8,0E-03	
C7^{o}	15.3 (16.5)	4.58 (4.4)	YYWVAVTNYGDEDNEGLR				C5KAM3	Pseudouridine synthase	4,0E-08	Protein synthesis
C7^{m}			YYWVAVTNYGDEDNEGLR				C5KAM3		4,0E-08	
C7^{ch}			YYSVVAVTNYGDEDNEGLR				C5KAM3		4,0E-08	
C13^{m}	37.0 (48.5)	6.6 (6.8)	FATLSFSTFGSAK			Phosphate acetyltransferase; *Clostridium acetobutylicum*	P71103	Phosphate acetyltransferase	1,60E-02	Metabolism
C13^{ch}			RYYNRTFLSFSTFGSAK			Putative uncharacterized protein; *Tetrahymena thermophila*	Q22KJ6		4,00E-02	
C14^{o}	13.81 (15.2)	5.5 (5.4)	SSAQEEVASFR				C5KKP3	Vacuolar ATPasa subnunit g	6,0E-04	Electron transport
C14^{m}			YWALQSTSGGEDEELAK				C5LSM9		5,0E-07	
O1	49.7 (57.0)	5.2 (4.9)	TLPLGLGTSSPLFK			Putative uncharacterized protein; *Tetrahymena thermophila*	Q239D4	Conserved hypothetical protein	3,0E-03	
O3	47.3 (66.0)	6.0 (6.8)	RPTELSQFLNNR			Putative uncharacterized protein; *Tetrahymena thermophila*	Q22MW9	Formate dh	3,0E-03	Electron Transport
O6	27.5 (36.0)	5.1 (5.4)	FLPGVNNQTAQGLLK			Proteasome subunit alpha type 4, subunit, putative; *Toxoplasma gondii*	B9QKC7	Proteasome subunit	3,0E-03	Proteolysis
O7	26.9 (36.0)	5.2 (5.5)	TGPGAYTGELTADFLK			Triosephosphate isomerase, putative; *Cryptosporidium muris*	B6AI68	Triosephosphate isomerase	2,0E-04	Carbohydrate metabolism
O14	17.0 (24.0)	6.3 (5.6)		K.ALGVDFDVTPVLGNVR.C	0.98	Peroxiredoxin V protein; *Perkinsus chesapeaki*	A7YXM7	Peroxiredoxin V	4,0E-11	Antioxidant/ Detoxification

Spot No.	Mw Theor./ Mw Exp.	pI Theor./ pI Exp.	PEAKS sequence	SEQUEST sequence	Sf	Identified protein; organism (automated search)	UNIPROT AC	Blast (manual search)	E-value	Function
M1	44.8 (58.0)	6.0 (6.6)		R.VDFNVPIKDGK.V	0.93	Phosphoglycerate kinase; *Euplotes crassus*	O02608	Phosphoglycerate kinase	3,0E-06	Carbohydrate metabolism
			TPLGNSLFDEEGSK			Phosphoglycerate kinase; *Toxoplasma gondii*	Q1KSE8		2,0E-07	
M4	28.8 (44.0)	6.7 (6.4)	FADGYLLSTGTPK			40S ribosomal protein S3, putative; *Theileria annulata*	Q4U9P5	40S Ribosomal Protein S3	8,0E-06	Protein synthesis
Ch1	30.9 (60.0)	4.6 (4.7)	RANHFFVSSAVDR			Phosphodiesterase, putative; *Toxoplasma gondii*	B9QPB2	Conserved hypothetical protein	3,0E-04	
			YVGDDALTAR			Actin 1-9; *Paramecium tetraurelia*	Q3M0W9		7,0E-04	
Ch6	29.9 (38.0)	5.0 (6.1)	YTTLLDGAADTVNLLK			Putative uncharacterized protein; *Plasmodium vivax*	A5K1C2	Conserved hypothetical protein	2,0E-07	
Ch7	25.6 (29.0)	5.5 (6.1)	VDEFLGLVTDVR			Putative uncharacterized protein; *Cryptosporidium muris*	B6ABM4	Glutathione S-transferase	3,7E-03	Antioxidant/ Detoxification
Ch8	21.6 (28.5)	5.9 (6.1)	YADVLLEDDGVAVR			Peroxiredoxin TSA1; *Saccharomyces cerevisiae*	P34760	Peroxiredoxin II	1,0E-04	Antioxidant/ Detoxification
Ch9	22.2 (24.0)	4.9 (4.6)	VLDFLDDELPR			Chromosome undetermined scaffold_79, *Paramecium tetraurelia*	A0E4U1	Conserved hypothetical protein	2,0E-03	
Ch11	17.0 (17.2)	6.3 (5.2)		K.ALGVDFDVTPVLGNVR.C	0.98	Peroxiredoxin V protein; *Perkinsus chesapeaki*	A7YXM7	Peroxiredoxin V	4,0E-11	Antioxidant/ Detoxification
			YYEFDFRRDFDVTPVLGNVR			Peroxiredoxin V protein; *Perkinsus chesapeaki*	A7YXM7		6,0E-05	
Ch12	17.0 (17.2)	6.3 (5.8)	WAVGDALPNVTVR			Putative uncharacterized protein; *Cryptosporidium hominis*	Q5CGM7	Peroxiredoxin V	2,0E-04	Antioxidant/ Detoxification

VI.5. DISCUSSION

Around 2000 protein spots were discriminated from each of three species of the genus Perkinsus: *P. olseni*, *P. marinus* and *P. chesapeaki* by 2DE. The results showed that *P. marinus* and *P. olseni* are closer between them that each one with *P. chesapeaki*. That agrees with the report of Moss et al. (2008) who analysed inter-specific genetic distance (uncorrected 'p') within the genus *Perkinsus*, according to the sequence of three regions of the DNA of *Perkinsus* spp., the internal transcript space (ITS) of the rDNA gene, the large subunit (LSU) of the rDNA gene and actin genes.

Limited funding restricted the attempts to identify separated spots to just 84 spots, from which 42 were sequenced and 28 were annotated to a known protein. If the experiment had be done after Joseph et al. (2010) had sequenced *P. marinus* genome, the number of identified proteins could have been higher because, with that information, a simpler and cheaper approach for protein sequencing had been used, avoiding *de novo* sequencing, such as peptide mass fingerprinting. The identified proteins were classified into 8 categories, according to GO classification. Six spots corresponded to two proteins implicated in electron transport: Formate dehydrogenase and vacuolar ATPase (V-ATPase) b and g subunit. The Formate dh protein is involved in the catalisation of the oxidative decarboxilation of malate to piruvate. This protein is ubiquitous in eukaryotes with different isoforms in cytosol, mitochondrion and chloroplast (Sánchez et al., 1996). The spots corresponding to V-ATPase consist of two large multimeric domains (one membrane spanning and one intracellular), each of which is composed by several subunits (Crider et al., 1997; Forgac, 1989). These enzymes function as proton pumps in a wide variety of cellular membranes, including endosomes, lysosomes, Golgi-derived vesicles, secretory vesicles and the plasma membrane of various cell types (Forgac, 2007). The b subunit of this enzyme was present in cells of the three species. The difference between the theoretical molecular mass (55.6 kDa) and the experimental one (94 kDa) could be due to an intermolecular disulphide bond formation. Previous reports have established the presence of a functional V-ATPase in the plasma membrane of Apicomplexan parasites with an important role in the regulation of their intracellular pH (Hayashi et al., 2000; Moreno et al., 1998). Additionally, V-ATPase plays an important role in chloroquine (antimalarian drug) accumulation in *Plasmodium falciparum* (Karcz et al., 1994). Subsequently, a polypeptide of 56 kDa was recognised and identified as vacuolar ATPase b subunit. This subunit lacks a signal sequence and the host cell targeting signal recently identified in the N terminus of many exported protein (Hiller et al., 2004; Marti et al., 2004), implying a different mechanism for targeting this protein to the plasma membrane of the host cell (Marchesini et al., 2005). This enzyme has also been found in *Toxoplasma gondii* tachyzoites (Moreno et al., 1998) and all developmental stages of *Trypanosoma cruzi* (Ulrich et al., 2011). We also detected the presence of V-ATPase g subunit in *P. olseni* and *P. marinus*.

Another important group of identified proteins consists of proteins involved in antioxidant/detoxification functions. Among these, peroxiredoxins deserve particular attention. These enzymes belong to the peroxidase family, which is found in different organisms including yeast, metazoans and protozoans. In some parasites as *Entamoeba histolytica* (Tachibana et al., 1991; Torian et al., 1990), *Fasciola hepatica* (McGonigle et al., 1997), and *Schistosoma japonicum* (Kumagai et al., 2006), peroxidases are characterized as antigens or secreted proteins, suggesting that these molecules may also participate in the defence against the host attacks (Rhee et al., 2005). Moreover, they have a role in receptor signalling, protein phosphorylation, transcriptional regulation and phagocytosis (Son et al., 2001). In this study, peroxiredoxin II was found in *P. chesapeaki* and peroxiredoxin V was found in *P. chesapeaki* and *P. olseni*, with differences in molecular weight and isoelectric point between the two species; these differences could be due to the presence of different isoforms of peroxiredoxin V. Changes in protein isoforms likely represent a range of biological activities, and may arise in numerous ways, for example, modification by glycosylation (Montes-Sanchez et al., 2009), phosphorylation (Barberis et al., 2009), or proteolitic process (Kilpatrick et al., 2009). *T. cruzi* expresses at least two distinct peroxiredoxin proteins with different subcellular localisations (Wilkinson et al., 2000), while three peroxiredoxins are found in *T. gondii* (Akerman and Müller, 2005) and four in *P. falciparum* (Kawazu et al., 2008). Several *P. marinus* oxidative pathway components that are expressed in the trophozoite stage have been identified, peroxiredoxin V among them (Joseph et al., 2010). *P. marinus* is able to inhibit the host production of reactive oxygen intermediates (ROIs) (Anderson, 1999; Volety and Chu, 1995) and to resist them (Schott et al., 2003); peroxiredoxins could be involved in the *Perkinsus* spp. mechanisms of avoiding ROIs produced by hosts. Glutathione S-transferase (GST), which was identified in *P. chesapeaki* in this study, is a part of the repertoire of adaptive response mechanisms to chemical stress in eukaryotic cells (Norppa, 2003) and its activity in aquatic organisms has been exploited as a biomarker of pollution (Myrnes and Nilsen, 2007). In *Perkinsus* spp., GST also could play an important role in protecting cells against the toxic products of lipid peroxidation that are implicated in the cytotoxicity of ROIs produced by the host (O'Leary et al., 1992; Sterbauer et al., 1988).

Another set of proteins identified in this study belongs to the protein synthesis category. Within this group we found Pseudouridine synthase, a common protein of the three *Perkinsus* spp., and 40S Ribosomal protein S3, protein exclusively detected in *P. marinus* and responsible for the most abundant posttranscriptional modification of cellular RNAs. These enzymes catalyse the site-specific isomerisation of uridine residues that are already part of an RNA chain, and appear to employ both sequence and structural information to achieve site specificity (Hamma and Ferré-D´ Amare, 2006; Ofengand, 2002). Anderson et al. (2009) described a pseudouridine synthase homologue that is central to the process of *Toxoplasma* differentiation, and suggested

a broadly conserved biological role of this enzyme beyond the simple biochemical action of modifying a nucleobase. Therefore, this enzyme could also be involved in cellular differentiation of the three species of *Perkinsus*.

The carbohydrate metabolism was represented by malate dehydrogenase, phosphoglycerate kinase and triose phosphate isomerase. These enzymes are involved in glycolysis and the tricarboxylic acid cycle pathway, all involved in the catabolism of glucose. Some studies have proposed the triose phosphate isomerase as a target for drug design in *E. histolytica* (Rodriguez-Romero et al., 2002) and phosphoglycerate kinase in *Trypanosoma brucei* (Bernstein et al., 1996). Pradhan et al. (2009) showed that malate dehydrogenase is mainly distributed throughout cytosolic compartment of *P. falciparum* and plays an important role in transferring and reducing equivalents across membranes. The signal transduction category was represented by the receptor of activated C kinase (RACK). This protein participates in important cellular processes such as cell cycle control, RNA processing (Neer et al., 1994) and acting as scaffold proteins that approximate enzymes and their substrates in specific subcellular sites (Schechtman and Mochly-Rosen, 2001). This study identified the RACK in *P. olseni*, *P. marinus* and *P. chesapeaki*. Previous works have established the presence of this receptor in *P. falciparum* (Madeira et al., 2003), *T. gondii* (Moran et al., 2007), *Trypanosoma carasii* (Ruszczyk et al., 2008) and *Leishmania mexicana* (Gómez-Arreaza et al., 2011). RACK orthologs in Trypanosomatidea have been postulated to play a role in controlling cellular division, including protozoan apoptosis (Schechtman and Mochly-Rosen, 2001) and as novel antigens for future vaccination studies (Ruszczyk et al., 2008). *P. falciparum* expresses one ortholog of RACK1, termed PfRACK (Madeira et al., 2003), that interacts with mammalian inositol triphosphate (IP_3) receptors in order to modulate the Ca^{+2} release from the host cell´s endoplasmatic reticulum. Such an effect could provide a mechanism for the parasite to directly manipulate the Ca^{+2} signalling machinery of host cells, thus providing a potential route to control host cell processes such as apoptosis, metabolism and cell proliferation, and to assure availability of nutrients to the intracellular parasite (Pusl et al., 2002; Rodrigues et al., 2007; Sartorello et al., 2009). Therefore, RACK could play similar roles in *P. olseni*, *P. marinus* and *P. chesapeaky*, but further studies are necessary to confirm this hypothesis.

A protein sequence matching that of a proteasome subunit from *P. marinus* was detected in *P. olseni*. The proteasome is involved in cell differentiation and replication in protozoan parasites such as *Entamoeb*a, *Trypanosoma*, *Plasmodium* and *Toxoplasma* spp., and thus it could be a therapeutic target. Proteasome are also involved in stress response, metabolic adaptation and cellular immune responses. The blockade of proteasome function prevent stage-specific morphological changes in *Trypanosoma*, *Plasmodium* and *Entamoeba*, and replication of *Plasmodium*, *Toxoplasma* and *Trypanosoma*, but not host cell entry by *Trypanosoma*, *Plasmodium* or *Toxoplasma* (Paugam et al., 2003). Further studies are needed to confirm that blockade of proteasome could inhibit the proliferation of *P. olseni*.

The low number of identified proteins makes difficult to find clear differences between the three *Perkinsus* spp. in terms of virulence, plasticity, infection mechanisms, pathogeny or host preference, thus the goals of the experiment were not totally achieved. Considering just the identified proteins, the proportion of proteins linked to carbohydrate metabolism and electron transport was higher in *P. olseni* and *P. marinus* than in *P. chesapeaki*, which could be linked to the higher *in vitro* proliferation rate of *P. olseni* and *P. marinus* with regards to that of *P. chesapeaki* (Cao et al., 2009a). The proportion of antioxidant/detoxification proteins was higher in *P. chesapeaki* than in the other two *Perkinsus* spp., which could be linked to a different way of interaction with the host immune system.

In conclusion, a representative 2DE map of cytosolic proteins of *P. olseni*, *P. marinus* and *P. chesapeaki* was established. The lack of sequences in databases allowed identifying only 13 different proteins, most of them clustered into electron transport, oxidative stress and detoxification, protein synthesis, carbohydrate metabolism, signal transduction, metabolic process and proteolysis. This is a step towards the generation of proteome profiles for use in future studies on protein expression, regulation and mechanisms of action under specific environmental conditions. Protein data together with the provision of the genome sequence for *P. marinus* (Joseph et al., 2010) and the applications of the genomic technologies should provide advances in the understanding of the biology of these parasites and the identification of key factors for virulence and infectivity.

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VII. PROTEIN EXPRESSION PROFILING IN HAEMOCYTES OF THE MANILA CLAM *Ruditapes philippinarum* IN RESPONSE TO INFECTION WITH *Perkinsus olseni*

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VII.1. ABSTRACT

The protein expression profiling of clam haemocytes in response to *P. olseni* was addressed. Clams from a *P. olseni*-free bed were challenged with parasite zoospores to analyse early immune response. Additionally, effects of longer term infection were assessed by comparing moderate to very heavily-infected clams with non-infected ones. Haemocyte proteins were separated by two dimensional electrophoresis (2DE), spot patterns were compared between treatments and relevant spots were sequenced. High variability in the total number of proteins in 2DE gels was found but differences between treatments were not significant. Seven proteins of which expression was markedly affected by *P. olseni* were identified: heat shock 70 protein 12B, integrin-α PS3, cytochrome C oxidase I, actin and ankyrin-3 were affected by challenging with *P. olseni* while transcriptional regulator ATRX and rho GTPase-activating protein 6 were differentially expressed in clams with advanced infection with regard to non-infected clams after long-term field exposure to the parasite. Thus, rho GTPase-activating protein 6 could hypothetically be a marker of resistance against *P. olseni* infection.

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VII.2. INTRODUCTION

The Manila clam *Ruditapes philippinarum* is the most marketed clam species worldwide. The world production reached 5178 tonnes in 2009 (FAO 2013). After cockle fishery decline (Villalba et al., 2014), the Manila clam fishery has raisen as the most important shellfishery in Galicia (NW Spain) in terms of biomass; its production in 2013 was 1.9 tonnes (official records in www.pescadegalicia.com). *R. philippinarum* was introduced into European coast for aquaculture purposes (Gosling, 2003); its introduction probably brought new diseases for European clams, such as infection with *Perkinsus olseni* (Chapter III). This protistan parasite has been blamed for Manila clam high mortality in Korea (Park and Choi, 2001; Choi and Park, 2010), China (Liang et al., 2001; Wu et al., 2011), Italy (Pretto et al., 2014) and Spain (Santmartí et al., 1995). The pathogenicity of this parasite in the Manila clam has been demonstrated (Shimokawa et al., 2010; Waki et al., 2012; Waki and Yoshinaga, 2013) and sublethal effects such as reduction of host fecundity (Park et al., 2006; Uddin et al., 2010; Casas and Villalba, 2012), may also involve economic losses. *P. olseni* has been reported infecting many other molluscan species worldwide, sometimes in association with host mortality (Villalba et al., 2004, 2011).

In Galicia, *P. olseni* is associated with mortality of the autochthonous clam *Ruditapes decussatus* (Villalba et al., 2005) but accurate measures of the effects of the parasite on Manila clam in this region have not been published thus far.

Haemocytes are the immune effector cells in molluscs; these cells are involved in inflammation, wound repair, encapsulation, phagocytosis, antimicrobial peptide secretion and oxidative enzymes production (Donaghy et al., 2009; Brulle et al., 2012). The interaction between the immune system of the Manila clam and *P. olseni* has been reviewed by Soudant et al. (2008, 2013) and for the clam involves increasing the number of circulating haemocytes, synthesis and secretion of specific lectins, encapsulating the parasite by host haemocytes and realising a specific polypeptide from encapsulating haemocytes (Montes et al., 1995a, 1995b). The clam immune response to *P. olseni* relies on changes of gene expression. Kang et al. (2006) analysed the gene expression of Manila clams infected with *P. olseni* and identified genes involved in the immune response against this parasite. Prado-Alvarez et al. (2009) identified differentially expressed genes in the carpet-shell clam *R. decussatus* against *P. olseni*. Complementary information to that obtained through genomic approaches could be provided by proteomics. In fact, proteomic approaches have been envisaged to identify proteins with a relevant role in the interaction of the oyster *Saccostrea glomerata* with the protistan parasite *Marteilia sydneyi* (Simonian et al., 2009a, 2009b), the oyster *Ostrea edulis* with *Bonamia ostreae* (Cao et al., 2009b), the scallop *Chlamys farreri* with *Vibrio* (Huan et al., 2011) and with the acute viral necrosis virus (Chen et al., 2011), and the mussel *Mytilus galloprovincialis* with pathogenic bacteria (Wu et al., 2013). In this study, a proteomic approach was implemented to identify

differentially expressed *R. philippinarum* haemocyte proteins linked to *P. olseni* infection. Early changes of the haemocyte protein composition due to infection were analysed by comparing clams from a *Perkinsus*-free area that were challenged in the laboratory with parasite zoospores *versus* non-challenged clams. Additionally, effects of longer term infection were assessed by comparing heavily-infected clams with non-infected ones, both collected from a *P. olseni*-affected bed.

VII.3. MATERIALS AND METHODS

VII.3.1. Clams and exposure to Perkinsus olseni

VII.3.1.1. Experimental challenge

A total of 110 clams (40-45 mm long) were collected from a natural bed in Camariñas (Galicia, NW Spain) that was considered as a *Perkinsus*-free bed (unpublished results). The absence of *Perkinsus* spp. was confirmed in 30 of those clams, which were diagnosed by PCR using the primers PerkITS85 and PerkITS750 (Casas et al., 2002a). The remaining 80 clams were used for an experimental challenge with *P. olseni* zoospores. We tried to mimic the natural infection path, thus we use zoospores because this is the most frequent infective stage in perkinsosis natural transmission (Ragone-Calvo et al., 2003) and we avoided injecting or inoculating the parasite into the clams. Forty clams were submerged in individual glasses containing a suspension of 10^6 *P. olseni* zoospores in 250 mL of filtered seawater with aeration, for 24 h. *P. olseni* zoospores had been obtained from carpet-shell clams *R. decussatus* heavily infected with *P. olseni*, following the procedure described by Casas et al. (2002a), and were collected immediately before the challenge. The remaining 40 clams were submersed in glasses without zoospores, as control. After challenge, clams were transferred to a 40 litter tank with aerated filtered seawater and kept for 8 days. Haemolymph was removed from the adductor muscle of each clam using a 25 gauge needle attached to a 1 mL syringe; a drop was examined with light microscope and the haemolymph samples contaminated with gametes or bacteria were discarded. The haemolymph was transferred into a 1.5 mL tube and kept on crushed ice until centrifugation (800 g, 10 min, 4 ºC). The supernatant containing the plasma was stored for further analysis and the pellet (cells) was frozen at -80 ºC until protein extraction. Two haemocyte pools, each deriving from the haemolymph of 20 challenged clams were produced and, similarly, two haemocyte pools were produced with control clams.

VII.3.1.2. Field exposure

Two hundred Manila clams *R. philippinarum* (40-45 mm long) were collected from a natural bed in Vilalonga, Ría de Pontevedra (Galicia, NW Spain), where clams

with high infection intensity were common (Villalba et al., 2005). On average, clams reach the minimum market size (40 mm) when they are around two years old in Galician rías, thus the collected clams had been naturally, long term exposed to *P. olseni*. Haemolymph was removed from the adductor muscle of each clam and haemocyte and plasma fractions were separated as described above. Then, each clam was opened and two gill lamellae were processed to diagnose intensity of *P. olseni* infection by the Ray's fluid thioglycollate medium (RFTM) method (Ray, 1966), using the intensity scale established by Mackin, from 0 (null infection) to 5 (very heavy infection) (Villalba et al., 2005). After diagnosis, three haemocyte pools from non-infected clams plus three haemocyte from infected clams were produced, each pool deriving from the haemolymph of 14 clams. Each infected pool was produced with haemocytes from six clams with moderate infection (score 3), 5 clams with heavy infection (score 4) and 3 clams with very heavy infection (score 5). Clams with very light (score 1) and light (score 2) infections were not used.

VII.3.2. Protein extraction

The haemocyte pools were resuspended in 1 mL of lysis buffer (8M Urea, 2M Thiourea, 2% CHAPS, 1% DTT, 0.8% Ampholites) for 2h 30 min at 4 °C, with vigorous shaking, for protein extraction. The protein concentration was determined by a Lowry Assay using the RC/DC Protein Assay Bio-Rad and measuring in a microplate lecture Expert 96 by Asys Hitech. Then, 250 µg of protein were purified using the 2D CleanUp kit Bio-Rad and resuspended in 300 µL of rehydration solution (7M urea, 2M thiourea, 4% chaps, 0.3% DTT, 0.5% IPG Buffer and bromophenol blue traces).

VII.3.3. Two dimensional electrophoresis (2DE) and image analysis

For Isoelectro Focusing (IEF), aliquots of 250 µg of haemocyte protein were applied onto IPG strips (17cm, pH 5-8, Bio-Rad) diluted in 350 µL of rehydration buffer. After 6-h passive and 6-h active (50V) rehydration, Iso-Electro Focusing (IEF) was performed (20 ºC, 50 µA/strip) in a Protean®IEF System from Bio-Rad through six steps: 500 V, 90 min; 1000 V, 90 min; 2000 V, 90 min; 4000 V, 90 min; 8000 V, 120 min, and 8000 V until 60000 Vh (4 h). After IEF, the strips were equilibrated in two steps of 20 min each, in equilibration buffer; 10 mg/mL DTT were used in the first step to reduce proteins and 45 mg/mL iodoacetamide were used in the second step to alkylate proteins. The proteins on the equilibrated IPG strips were separated across 12.5% SDS–PAGE gels using a vertical system (PROTEAN Plus Dodeca Cell, Bio-Rad) and standard Tris/glycine/SDS buffer. Gels were run at 2.5 W/gel for 15 min followed by 12.5 W/gel for 5 h 30 min. Molecular weight markers (BioRad) were run in the second dimension next to the problem sample for protein size determination. Four gels were produced from each haemocyte pool, rejecting the worst of them thus keeping three replicates.

The gels were silver stained with a protocol compatible with MS analysis: (1) incubation in 50% (v/v) methanol 5% (v/v) acetic acid for 1 hour or overnight; (2) incubation in 50% methanol for 30 min; (3) wash with H_2O milli-Q for 15 min (x 2); (4) incubation in 0.02% (w/v) sodium thiosulfate for 1 min; (5) wash with H_2O milli-Q for 1 min (x2); (6) incubation in 0.1% (w/v) silver nitrate for 30 min; (7) wash with H_2O milli-Q for 1 min (x3); (8) incubation in 0.04% (v/v) formaldehyde 2% (w/v) sodium nitrate until complete appearance of the spots; (9) incubation in 1% (m/v) EDTA for 5 min to stop staining. Once stained, the gels were digitised by a GS-800 densitometer scanner (BioRad). The protein patterns were analysed using PD Quest 8.4.0 software (Bio-Rad). High reproducibility was observed in the replicate gels of each treatment in terms of number of spots, position and intensity. A master gel of each treatment was produced including just the spots shared by all the gels of the treatment. Then spot patterns were analysed and compared between treatments (challenged vs. control clams in the experimental challenge and infected vs. non-infected clams in the field exposure), searching for differences in protein expression profiles, specifically to identify the spots shared by master gels and spots exclusive of each master gel. The gels were calibrated using a select set of reliable identification landmarks distributed throughout the entire gel to determine isoelectric point (*pI*) and molecular weight (Mw) coordinates for each single spot. Percentages of similitude between gels were calculated as the number of common spots shared by two gels with regard to the total number of spots:

$$PS = [C \times 2 / (T1 + T2)] \times 100$$

where PS is the percentage of similitude, C is the number of the common spots shared by the 2 gels, and T1 and T2 are the total number of spots in region 1 and 2, respectively.

VII.3.4. Protein identification and database search

A number of spots in the gels were selected for sequencing; those spots were manually excised from the silver stained gels and sent to the proteomics service of the Ramón Dominguez Foundation (Complejo Hospitalario Universitario de Santiago de Compostela, Spain) for sequencing. A mass spectrometry approach as matrix-assisted laser-desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) was used in protein identification of trypsin digested spots (Shevchenko et al., 1996). MS data were obtained in an automated analysis loop using 4800 MALDI-TOF/ TOF analyser (Applied Biosystems). Peptide mass fingerprinting (PMF) and peptide fragmentation spectra data of each sample were combined through the GPS Explorer Software v3.6 (Applied Biosystems) using Mascot software v2.1. (Matrix Science, London, U.K.) to search against the non-redundant protein database of Mollusca taxa included in the NCBI (National Center for Biotechnology Information, Maryland, USA).

Due to the poor protein and DNA sequence database coverage for *Ruditapes philippinarum*, most proteins were identify by *de novo* sequencing and BLAST similarity searching following a procedure outlined by Liska and Shevchenko (2003). All MS/MS spectra for a sample were sequenced *de novo* using ProBLAST 1.4 software (Applied Biosystems) (Altschul et al., 1997), and the top six candidate sequences for each MS/MS combined into a single text-format search string. The search string was then submitted to MS BLAST sequence similarity searching using a server version of the Paracel BLAST software (Paracel, Pasadena, CA, U.S.). Protein identification significance was judged using the MS BLAST scoring algorithm. Additionally, fragmentation patterns calculated from the assigned peptide sequences were matched against raw MS/MS data using links provided by the Pro BLAST software. Only the proteins that matched with a minimum of three peptide sequences with at least 20 identical amino acid residues were included in the result list, providing more stringent criteria for the identification according to Jorge et al. (2005) procedure. Theoretical Mw and pI values of the identified proteins were obtained using the compute pI/Mw tool at the ExPASy Proteomics Server (http://www.expasy.org/tools/pi_tool.html).

VII.3.5. Statistics

Differences in the number of spots between gels from clams challenged with *P. olseni* zoospores and non-challenged clams, between gels from infected and non-infected clams (field exposure) and between gels from non-challenged and non-infected clams were analysed by ANOVA. Significance level was established at $P \leq 0.05$.

VII.4. RESULTS

Fig. VII.1 shows representative 2-DE gels of each treatment. More than 500 spots were counted in the gels, with an average of 570 spots per gel. Comparison between replicate gels from each treatment of the two experiments showed percentages of similitude above 60% (Table VII.1). The analysis of the gels is summarised in Table VII.2. The master gels included around 40% of the spots of the replicate gels of each treatment (Table VII.2). In the experimental challenge, the average number of spots in the gels of challenged clams was higher than in those of the control clams, while in the field exposure, the average number of spots in gels of infected clams was lower than in those of non-infected clams. However, differences in the number of spots between gels from clams challenged with *P. olseni* zoospores and non-challenged clams, those between gels from infected and non-infected clams (field exposure), and those between non-challenged and non-infected clams were not statistically significant (the probability values were 0.36, 0.64 and 0.25, respectively). The percentage of similitude between the master gel of challenged clams and that of non-challenged clams was 61.2%, while for the field exposure experiment, the

percentage of similitude between the master gel of infected clams and that of non-infected clams was 67.8%. The two pair comparisons between treatments (challenged vs. control clams in the experimental challenge and infected vs. non-infected clams in the field exposure) allowed to detect spots shared by the two treatments of each comparison and spots exclusive of a treatment (Table VII.2). The 11 spots that were found exclusive of a treatment were excised from the stained gels, sequenced and annotated; seven of them were found to correspond to known proteins. Information on sequenced spots is summarised in Table VII.3.

Table VII.1. Comparison of protein expression profiling between haemocyte pools from the same treatment in the two experiments: experimental challenge and field infection. For each treatment, diagonal cells show the number of spots shared by gel replicates from each pool. The number of spots shared between pairs of pools is shown in cells below the diagonal, and the percentage of similitude between pairs of pools is shown in cells above the diagonal.

Experiment	Treatment	# Pool	# Pool		
		# Pool	**1**	**2**	
Experimental challenge	Challenged	**1**	363	67.5%	
		2	252	383	
	Control	# Pool	**1**	**2**	
		1	448	65.3%	
		2	248	311	
Field exposure	Infected	# Pool	**1**	**2**	**3**
		1	577	62.2%	78.2%
		2	271	295	64.1%
		3	414	249	482
	Non-infected	# Pool	**1**	**2**	**3**
		1	539	81.3%	68.3%
		2	433	526	69.3%
		3	306	306	357

Table VII.2. Summary of information on spots in gels produced through 2DE with samples of proteins of *Ruditapes philippinarum* haemocytes, including the average number of spots in the replicate gels of each treatment, the number of spots in the master gel of each treatment, and the number of exclusive and shared spots in two pair comparisons between treatments: clams challenged with *P. olseni* zoospores vs. control clams in the experimental challenge and clams infected with *P. olseni* vs. non infected clams in the field exposure.

				Comparisons between treatments	
Experiment	**Treatment**	**Average no. spots in replicate gels**	**No. spots in master gels**	**No. exclusive spots**	**No. shared spots**
Experimental challenge	**Challenged**	596	252	3	153
	Control	561	248	5	
Field exposure	**Infected**	529	241	2	176
	Non-infected	592	278	1	

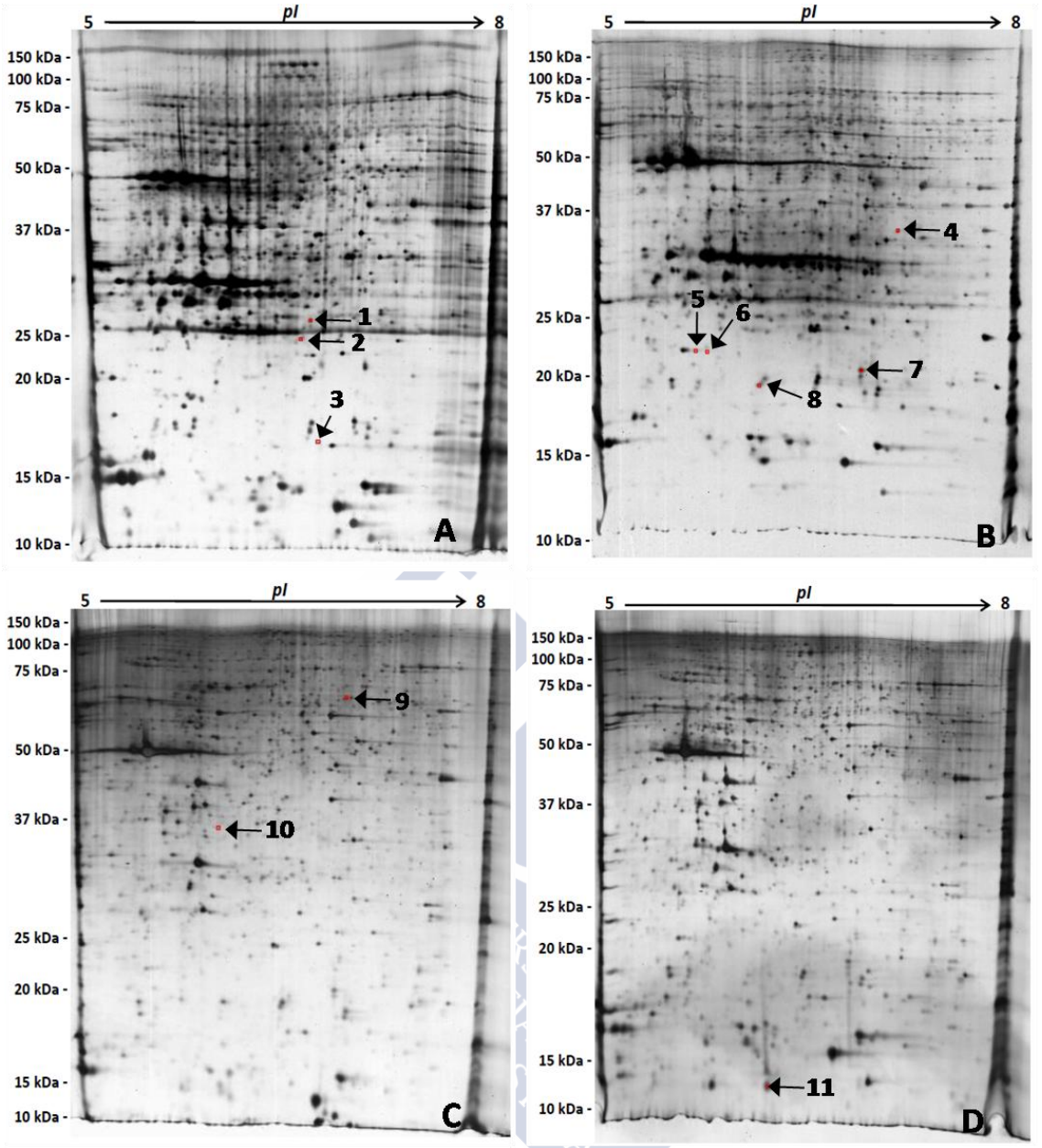

Fig. VII.1. Representative 2-D gels pattern of proteins of *Ruditapes philippinarum* haemocytes corresponding to the four treatments. A: Clams challenged with *Perkinsus olseni* zoospores. B: Control clam in the experimental challenge. C: Clams infected with *P. olseni* in field exposure. D: Non-infected clams in field exposure. Black arrows show exclusive spots of each treatment.

Table VII.3. Information on peptide sequence and protein annotation corresponding to the 11 spots excised from gels. Treatments: clams *Ruditapes philippinarum* challenged with *Perkinsus olseni* zoospores vs. control clams in the experimental challenge and clams infected with *P. olseni* vs. non infected clams in the field exposure. Mw obs/theo: the molecular observed in gels and the theoretical one, respectively. pI obs/theo: the isoelectric point value observed in gels and the theoretical one, respectively. Match/(% Cov): number of amino acids of the protein sequence that corresponds to matched peptides and percentage. Score: score of the match of peptides with proteins in database after BLAST searching; the higher the value of the score, the better the peptide match. E-value: expected value, which describes the number of hits expected to see by chance when searching a database of a particular size; the lower the E-value, the better the peptide match. Sequence: only the best match sequence is shown in the table. Protein identification: protein name of identified peptides in the database. Accession number: accession number of proteins in the database.

Code	Treatment	Mw obs/theo	pI obs/theo	Match/ (%cov)	Score	E-value	Sequence	Protein identification	Accession number
1	Challenged	27,4/18,12	6,4/9,86	11/15 (73%)	29,1	0,057	TMDKVTNSPGSSPAR	Hypothetical protein CGI_10027008 [*Crassostrea gigas*]	EKC36473.1
2	Challenged	25,8/64,88	6,3/6,84	7/7 (100%)	26,5	0,2	VVMEDGP	Heat shock 70 kDa protein 12B [*Crassostrea gigas*]	EKC42407.1
3	Challenged	18,4/110,47	6,4/5,35	10/14 (71%)	29,1	0,058	SGGEGSIKSSRGNT	Integrin alpha-PS3 [*Crassostrea gigas*]	EKC41508.1
4	Control	33,4/47,22	7,1/11,07	8/10 (80%)	28,2	0,078	IKARTGNEKM	Hypothetical protein CGI_10001747 [*Crassostrea gigas*]	EKC22674.1
5	Control	21,8/21,2	5,45/8,11	6/6 (100)	25,2	0,43	DMNFPR	Cytochrome oxidase c subunit I [*Mesodesma mactroides*]	ADH03916.1
6	Control	21,7/2,01	5,5/9,52	8/9 (88%)	26,9	0,039	AVFPSLVGR	Actin, partial [*Malletia abyssorum*]	AEM68335.1
7	Control	20,1/156,04	6,9/5,60	9/14 (64%)	29,5	0,043	MRTFECKKGVSEPR	Ankyrin-3 [*Crassostrea gigas*]	EKC42583.1
8	Control	19,5/40,04	6,1/6,61	10/17 (58%)	27,4	0,25	VPGPNRRLALYHSRGGH	Hypothetical protein CGI_10022803 [*Crassostrea gigas*]	EKC37219.1
9	Infected	62,4/211	6,65/6,21	8/9 (88%)	29,9	0,021	FTANMPNMG	Transcriptional regulator ATRX [*Crassostrea gigas*]	EKC43176.1
10	Infected	34,7/82,53	6,1/6,11	8/9 (88%)	27,8	0,098	GGPPIDVIE	Hypothetical protein CGI_10017740 [*Crassostrea gigas*]	EKC33588.1
11	Non-infected	14,1/110,15	6,3/8,47	9/11 (81%)	46,2	0,034	SAKGENKDVII	Rho GTPase-activating protein 6 [*Crassostrea gigas*]	EKC35433.1

VII.5. DISCUSSION

Results demonstrate that *P. olseni* induces differential protein expression in haemocytes of the Manila clam *R. philippinarum*. The high variability in the total number of spots in gels between replicate gels of each pool involved that differences in that variable between treatments were not significant. Our experimental approach only allowed detecting qualitative differences of protein expression; quantitative differences could not be measured although they had to occur. Many host physiological processes show temporal variation associated with infection progression, even variations in the number of circulating haemocytes and of those infiltrated in the connective tissue of host organs affected by *P. olseni* (Casas, 2002a; Soudant et al., 2008, 2013). A peak of expression short time after challenge followed by drop was reported in immune related genes in haemocytes of clams *R. decussatus in vitro* challenged with *P. olseni* zoospores (Prado-Alvarez et al., 2009). A substantial reduction of proteins in the whole haemolymph of oysters *Ostrea edulis* infected with *Bonamia ostreae* with regard to non-infected oysters was reported by Cao et al. (2009b). Our conservative approach, which involved just considering the spots shared by all the replicates of each treatment and detecting qualitative but not quantitative differences between treatments, resulted in finding scarce but robust differences in the haemocyte protein profiling between treatments. Thus, in a proteomic context, more discriminating approaches, such as quantitative analysis of spot patterns in gels or shotgun proteomics, could be used to further study Manila clam-*P. olseni* interaction.

Remarkable discrepancies were found between the theoretical values of molecular weight and/or isoelectric points of some of the identified proteins and the observed values according to the position of the spots in the gels. These discrepancies could correspond to isoforms generated by a variety of causes, including post-translational modifications, alternative splicing and the occurrence of multigene families (De La Fuente et al., 2011). The sequence of four excised spots matched non-identified proteins of *Crassostrea gigas* included in databases. The sequence of the remaining seven excised spots matched identified proteins. Two spots occurring exclusively in gels of haemocytes of clams challenged with *P. olseni* zoospores matched the heat shock 70 protein 12B and integrin-α PS3. Heat shock proteins act as molecular chaperones, facilitating proper folding, unfolding, assembly and intracellular transport of proteins. Heat shock proteins in the 70 kDa family (HSP70) are the most commonly expressed heat shock proteins in response to stress (Lindquist, 1992). HSP response also plays a significant role in regulation of the immune response; in mammals they have a range of roles in the inflammatory process such as mediating cytokine production and activating macrophages (Roberts et al., 2010). HSP70 proteins consist of two functional domains: the highly conserved ATPase domain of 44-kDa in the N-terminus and the substrate binding region of 25-kDa in the C-terminus (Steagall et al.,

2006); in our case, probably only the 25-kDa subunit was sequenced, which would explain the molecular weight of the spot observed in the gel. The involvement of HSP70 in bivalve mollusc immune response had been demonstrated by assessing increased HSP70 gene expression after challenge with pathogenic bacteria in *Mytilus galloprovincialis* (Cellura et al., 2006), *Argopecten irradians* (Song et al., 2006), *R. decussatus* (Gestal et al., 2007), *Pinctada fucata* (Wang et al., 2009) and *R. philippinarum* (Moreira et al., 2014).

Regarding integrin-α PS3, in some invertebrates such as *Drosophila*, integrin is formed by three different heterodimers from the combination of three α subunits: αPS1, αPS2, αPS3 and one β subunit (Brower, 2003). Integrin is a glycoprotein involved in haemocyte encapsulating response as well as in phagocytosis of apoptotic cells and bacteria in several invertebrates (Pech and Strand, 1995; Terahara et al., 2006; Zhuang et al., 2008; Nonaka et al., 2013). Integrins play important roles in a variety of biological phenomena by mediating cell-cell adhesion, connecting the extracellular matrix with the cytoskeleton and activating intracellular signalling pathways (Margadant et al., 2011; Nonaka et al., 2013). Integrins are capable of inducing phagocytosis, probably due to their ability to remodel the cytoskeleton, and targets for integrin-mediated phagocytosis include apoptotic cells and microorganisms (Wu et al., 2006; Dupuy and Caron, 2008). This mechanism of action is sometimes exploited by microorganisms to gain entry into host cells (Dupuy and Caron, 2008). In *C. gigas,* Terahara et al. (2006) found that the expression level of integrin was significantly higher in hyalinocytes than in granulocytes and agranulocytes, suggesting that phagocytosis of hyalinocytes is regulated by integrin-dependent mechanism and that of granulocytes is elucidated by other functional receptors. The main haemocyte reaction of clams against *P. olseni* infections is the encapsulation (Montes et al., 1995a; Soudant et al., 2008, 2013), which is consistent with our detection of integrin-α PS3 in clam haemocytes after challenging clams with *P. olseni* zoospores.

Three spots occurring exclusively in gels of haemocytes of control clams in the experimental challenge matched cytochrome C oxidase I, actin and ankiryn-3; in other words, *P. olseni* should be the cause of the absence of these proteins in the gels from haemocytes of challenged clams. Cytochrome C oxidase I is the main subunit of the cytochrome c oxidase complex (COX), which is a large transmembrane protein complex catalysing the final step in mitochondrial electron transfer chain; it is regarded as one of the major regulation sites for oxidative phosphorylation. Link of COX with immune response can be deduced by the fact that COX inhibition leads to energy depletion, excessive production of reactive oxygen species and apoptosis (Hüttemann et al., 2012). It is difficult to explain the lack of the spot corresponding to cytochrome C oxidase I in the gels of haemocytes from challenged clams because of the importance of COX for cell survival; perhaps the spot corresponded to an isoform. The procedure for protein extraction from haemocytes is highly efficient for cytosolic proteins but less efficient for membrane proteins, thus the spot could correspond to cytochrome C

oxidase I non-linked to membrane, which would be much less abundant in haemocytes of challenged clams. Cytochrome C oxidase I has been involved in previous puzzling reports: it was included both in up-regulated and down-regulated gene libraries of haemocytes of *R. decussatus* challenged with *P. olseni* (Prado-Alvarez et al., 2009) and of *O. edulis* challenged with *B. ostreae* (Martín-Gómez et al., 2012).

Actin is included in the cytoskeleton, which involves direct implication in cell motility. Thus, actin participates in many important cellular processes related to immune response, such as phagocytosis, encapsulation, cell signalling, and vesicle and organelle movement (Chen et al., 2011). Actin may have multiple isoforms with different values of isoelectric point and molecular weight, probably due to posttranslational modifications (Zhang et al., 2010); some of the actin isoforms have been shown to be down-regulated in the scallop *Chlamys farreri* response to acute viral necrosis virus (Chen et al., 2011), as well as in shrimps in response to white spot syndrome virus (Wang et al., 2007), yellow head virus (Bourchookarn et al., 2008) and *Vibrio anguillarum* (Zhang et al., 2010), while others did not change. The down-regulation of actin may be common in the response of marine invertebrates to pathogens (Chen et al., 2011).; this could be the case of *P. olseni*, which could have been responsible for the lack of this actin spot in the gels of haemocytes from challenged clams trying to evade haemocyte attack.

Ankyrin-3 is included in a family of proteins, ankyrins, that are believed to link the integral membrane proteins to the underlying spectrin-actin cytoskeleton and play key roles in activities such as cell motility, activation, proliferation, contact, and the maintenance of specialised membrane domains. Multiple isoforms of ankyrin with different affinities for various target proteins are expressed in a tissue-specific, developmentally regulated manner. Most ankyrins are typically composed of three structural domains: an amino-terminal domain containing multiple ankyrin repeats, a central region with a highly conserved spectrin binding domain, and a carboxy-terminal regulatory domain which is the least conserved and subject to variation (Hoock et al., 1997; Raphael et al., 2000; Bennett and Chen, 2001). Proteases of *Plasmodium falciparum* are involved in erythrocyte rupture, which possibly requires to breakdown erythrocyte membrane skeleton; thus, *P. falciparum* proteases cleave the membrane protein ankyrin to facilitate parasite release (Magowan et al., 2000; Raphael et al., 2000). The absence of those actin and ankyrin spots in clams challenged with *P. olseni* suggests that the parasite tries to impair haemocyte motility.

In the case of field exposure, the spot identified as transcriptional regulator ATRX was exclusive of infected clams, while the one identified as Rho GTPase-activating protein 6 was exclusive of non-infected clams. ATRX, is a member of SNF2 family of helicase/ATPases that is thought to regulate gene expression via an effect on chromatin structure and/or function (McDowell et al., 1999); it contains a highly conserved plant homeodomain (PHD)-like domain, present in many chromatin-associated proteins, and a carboxy-terminal domain which identifies it as a member of

the SNF2 family of helicase/ATPases (Gibbons et al., 2000). ATRX is involved in diverse cellular process, including DNA methylation, transcription, cell cycle and apoptosis (Lee et al., 2007). In humans, ATRX plays an important role in maintaining telomere integrity and likely facilitates normal telomere replication. Loss of ATRX protein by conditional gene-targeting in mice causes a 12-fold increase in neuronal apoptosis during early stages of corticogenesis (Lee et al., 2007). Infection with *Perkinsus marinus* reduces the normal apoptosis rate in *Crassostrea virginica* (Sunila and LaBanca, 2003); such effect has not been assessed in clams infected with *P. olseni* but the expression of transcriptional regulator ATRX in haemocytes of clams with an advanced *P. olseni* infection could contribute to keep haemocytes active to fight against the parasite.

Rho GTPase-activating protein 6 has significant connection with immune response because is involved in the regulation of rho family GTPases. The latter are intracellular signalling proteins regulating multiple pathways involved in cell actomyosin organization, adhesion, and proliferation; rho GTPase family are essential regulators of cell type specific functions and stimuli-specific pathways in haematopoiesis (Mulloy et al., 2010). Additionally, they are important components of signal transduction pathways used by antigen receptors, costimulatory, and cytokine and chemokine receptors to regulate the immune response (Cantrell, 2003). Highly pathogenic bacteria of the genus *Yersinia*, *Salmonella*, *Escherichia* and *Clostridium* exploit and/or impair many aspects of rho GTPase protein activities by activating or inhibiting these key molecular switches, to disrupt epithelial/endothelial barriers, paralyse immune cell migration and phagocytic functions, invade epithelial cells, replicate, and form reservoirs or disseminate in epithelia (Lemichez and Aktories, 2013). The lack of the spot corresponding to rho GTPase-activating protein 6 in gels from clams with advanced infection could mean that clams expressing that protein fight efficiently the parasite and infection does not progress; thus, rho GTPase-activating protein 6 could hypothetically be a marker of resistance against *P. olseni* infection, which deserves further assessment. Alternatively, it could suggest that important regulatory systems are impaired in the haemocytes of clams with advanced *P. olseni* infection including down-regulation of this protein. Wang et al. (2010) reported up-regulation of the gene of a rho GTPase-activating protein in the gills of oysters *C. virginica* after 30 days from challenge with *P. marinus*.

In conclusion, seven haemocyte proteins of which expression was markedly affected by *P. olseni* were identified; these proteins deserve further study to better understand Manila clam-*P. olseni* interaction and to assess their usefulness in clam perkinsosis management.

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VIII. PROTEIN EXPRESSION PROFILING IN PLASMA HAEMOLYMPH OF THE MANILA CLAM *Ruditapes philippinarum* IN RESPONSE *Perkinsus olseni* INFECTION

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VIII.1. ABSTRACT

The Manila clam *Ruditapes philippinarum* is susceptible to infection with the protistan parasite *Perkinsus olseni*. This study takes a proteomic approach to identify differentially expressed proteins in the clam plasma due to interaction with this parasite. Clams from a *P. olseni*-free bed were challenged with *P. olseni* zoospores to analyse short term effects in plasma protein profile. Longer term effects of infection were assessed by comparing moderate to very heavily infected clams with non-infected ones. Twenty five proteins occurring in plasma were found to be modulated after challenging clams with *P. olseni* zoospores, while no plasma protein was unequivocally found to be modulated by longer term *P. olseni* infection. Eighteen of those proteins could be sequenced from which 12 corresponded to known proteins. Some of the identified proteins, namely lysozyme and 3 lectins have a well-known role in clam immune response against the parasite; the others deserve further study to better understand Manila clam-*P. olseni* interaction.

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VIII.2. INTRODUCTION

Bivalve molluscs have an open circulation system and possess an innate immune system involving humoral factors and cell-mediated mechanisms (Soudant et al., 2013). The haemolymph is the blood of the molluscs, which includes haemocytes and plasma. Haemocytes are involved in the defence system of the molluscs, with the ability of ingesting or encapsulating and destroying microorganisms; haemocytes are chemotactic, mobile, phagocytic and able to aggregate (Soudant et al., 2013). The plasma includes the immune humoral factors released by haemocytes and other cells, such as lectins, antimicrobial peptides, lysozyme and protease inhibitors. Infection with the protistan parasite *Perkinsus olseni* triggers an inflammatory reaction in clams *Ruditapes philippinarum* and *Ruditapes decussatus*, involving mobilisation of haemocytes to encapsulate and destroy the parasite (Chagot et al., 1987). This defence reaction involves (1) chemotaxis, that is attraction and migration of haemocytes toward chemical stimuli released or carried by the parasite; (2) Recognition and attachment of haemocytes to parasite surface, mediated by lectins; and (3) Release of lysosomal hydrolytic enzymes and reactive oxygen species (ROS) from the haemocytes for the external degradation of the parasite (Soudant et al., 2013).

Various studies on the effects of infection with *Perkinsus* spp. on host humoral factors have been published, with some discrepancy among them. The protein concentration in the plasma of oysters *Crassostrea virginica* (highly susceptible to *Perkinsus marinus* infections) exposed to *P. marinus* was higher (Chu and La Peyre, 1993), lower (La Peyre et al., 1995) or similar (Chu and La Peyre, 1989) than/as in non-exposed oysters. Remarkably, the protein concentration in the plasma of oysters *Crassostrea gigas* (less susceptible to *P. marinus* infection) increased significantly after exposition to *P. marinus* (La Peyre et al., 1995). The plasma agglutination titre and the protein concentration in clams *R. philipinarum* naturally infected with *P. olseni* were higher than in non-infected clams, while no variation was detected in lysozyme concentration (Ordás et al., 2000). The lack of concordance among studies could be due to high variation among individuals and the influence of external factors, such as season, habitat, salinity or temperature, on the serum protein concentration (Chu and La Peyre, 1989; Ordás et al., 2000; Villalba et al., 2004). Protease inhibitory activity was found in plasma from *C. virginica* oysters (Faisal et al., 1998), this serine protease inhibitor was proposed to be involved in the elimination of the protozoan parasite *P. marinus* as it inhibited the parasite´s proteases (La Peyre et al., 2010). Xue et al. (2006, 2009) purified and characterised two serine protease inhibitor genes from *C. virginica* (cvSI-1; cvSI-2) showing a theoretical protein of 7.6 kDa for cvSI-1 and 7.2 kDa for cvSI-2. These two proteins inhibit the bacterial protease subtilisin and the subtilisin-like protease perkinsin from *P. marinus* as well as the proliferation of the parasite itself *in vitro* (La Peyre et al., 2010; Yu et al., 2011). A polypeptide of 225 kDa is specifically

released by haemocytes of *R. decussatus* and *R. philippinarum* against *P. olseni* infection (Montes et al., 1995b, 1996). Kang et al. (2006) described an antimicrobial peptide similar to mussel defensin in clams *R. philippinarum* infected with *P. olseni*. Two more antimicrobial peptides with similarity with myticin and mytilin AMP previously identified in *Mytilus galloprovincialis* (Mitta et al., 1999) were identified in *R. philippinarum* (Gestal et al., 2007). Various lectin types are induced in clams *R. philippinarum* after infection with *Perkinsus olseni* or bacteria as *Vibrio* spp. (Bulgakov et al., 2004, Kang et al., 2006, Kim et al., 2008a,b; Villalba et al., 2011; Moreira et al., 2012). The involvement of plasma lysozyme in mollusc defence against bacteria and its antiprotozoal activity was confirmed (La Peyre et al., 2004; Itoh et al., 2007; Li et al., 2009; Zhao et al., 2010; Ding et al., 2014).

Most immune humoral factors are proteins. A proteomic approach was implemented to identify differentially expressed proteins in the haemocytes and plasma of Manila clams *R. philippinarum* in response to infection with *P. olseni*. Changes in the early stages of infection were analysed by comparing clams from a *Perkinsus*-free area that were challenged in the laboratory with parasite zoospores *versus* non-challenged clams. Additionally, effects of longer term infection were assessed by comparing heavily-infected clams with non-infected ones, both collected from the same *P. olseni*-affected bed. The results corresponding to changes in haemocyte protein profiling have been reported in the chapter VII. Results corresponding to plasma protein profiling are reported in this chapter.

VIII.3. MATERIALS AND METHODS

VIII.3.1. Clams and exposure to Perkinsus olseni

VIII.3.1.1. Experimental challenge

Clam origin, experimental exposition of clams to parasite zoospores, haemolymph collection, and separation of haemocytes from plasma are explained in the section VII.3.1.1. Two plasma pools, each deriving from the haemolymph of 20 challenged clams were produced and, similarly, two plasma pools were produced with control clams.

VIII.3.1.2. Field exposure

Clam origin, diagnosis and estimation of intensity of infection with *P. olseni*, haemolymph collection, and separation of haemocytes from plasma are explained in the section VII.3.1.2. Plasma pools were produced as explained for haemocyte pools in the section VII.3.1.2.

VIII.3.2. Protein extraction

The plasma pools were resuspended in 2 mL of lysis buffer (8M Urea, 2M Thiourea, 2% CHAPS , 1% DTT, 0.8% Ampholites, and 100mM phenylmethylsulphonyl fluoride, in order to avoid the protease activity in the sample) for 2 h 30 min at 4 °C, with vigorous shaking, for protein extraction. The protein concentration was determined by a Lowry Assay using the RC/DC Protein Assay Bio-Rad and measuring in a microplate lecture Expert 96 by Asys Hitech. Then, 500 µg of protein were purified using the 2D CleanUp kit Bio-Rad and resuspended in 400 µL of rehydration solution (7M Urea, 2M Thiourea, 4% CHAPS, 0.3% DTT, 0.5% IPG Buffer and bromophenol blue traces).

VIII.3.3. Two dimensional electrophoresis (2DE) and image analysis

For the Isoelectro Focusing (IEF), aliquots of 500 µg of plasma protein were applied onto IPG strips (17cm, pH 5-8, Bio-Rad) diluted in 350 µL of rehydration buffer. After 12 h active (50V) rehydration, IEF was performed (20 °C, 50 µA/strip) in a Protean®IEF System from Bio-Rad through three steps: 250 V, 15 min; 10000 V, 180 min and 10000 V until 60000 Vh (4h).

The second dimension involved separation in SDS-PAGE, which was performed through 12,5% polyacrylamide homemade gels, using a Protean®II xi cell (Bio-Rad), as described in the section VII.3.3. Four gels were produced from each plasma pool, rejecting the worst of them thus keeping three replicates. The gels were silver stained as described in the section VII.3.3. Once stained, the gels were digitised by a GS-800 densitometer scanner (BioRad). The protein patterns were analysed using PD Quest 8.4.0 software (Bio-Rad). High reproducibility was observed in the replicate gels of each treatment in terms of number of spots, position and intensity. A master gel of each treatment was produced including just the spots shared by all the gels of the treatment. Then, spot patterns were analysed and compared between treatments (challenged vs. control clams in the experimental challenge and infected vs. non-infected clams in the field exposure), searching for differences in protein expression profiles, specifically to identify the spots shared by master gels and spots exclusive of each master gel. The gels were calibrated using a select set of reliable identification landmarks distributed throughout the entire gel to determine isoelectric point (*pI*) and molecular weight (Mw) coordinates for each single spot. Percentages of similitude between gels were calculated as described in the section VII.3.3.

VIII.3.4. Protein identification and database search

A number of spots in the gels were selected for sequencing; those spots were manually excised from the silver stained gels and sent to the proteomics service of the Ramón Dominguez Foundation (Complejo Hospitalario Universitario de Santiago de

Compostela, Spain) for sequencing and protein identification as explained in the section VII.3.4.

VIII.3.5. Statistics

Differences in the number of spots between gels from clams challenged with *P. olseni* zoospores and non-challenged clams, between gels from infected and non-infected clams (field exposure) and between gels from non-challenged and non-infected clams were analysed by ANOVA. Significance level was established at $P \leq 0.05$.

VIII.4. RESULTS

Fig.VIII.1 shows representative 2-DE gels of each treatment. More than 340 spots were counted in the gels corresponding to the experimental challenge gels while more than 150 spots were counted in the gels corresponding to field infection, with an average of 389 and 174 spots respectively. Differences in the number of spots between gels from clams challenged with *P. olseni* zoospores and non-challenged clams and those between gels from infected and non-infected clams (field exposure) were not significant; however, differences between gels from non-challenged and non-infected clams were significant. Comparison of replicate gels from the two experiments showed percentages of similitude between 57 and 70% (Table VIII.1).

The analysis of the gels is summarised in Table VIII.2. The master gel included around 40% of the spots in the replicate gels of each treatment except in the case of non-infected clams from field infection, in which only 28% of the spots were represented (Table VIII.2). The percentage of similitude between the master gel of challenged clams and that of non challenged clams was 55.7%, while for the field exposure experiment, the percentage of similitude between the master gel of infected clams and that of non-infected clams was 74.79%. The two pair comparisons between treatments (challenged vs. control clams in the experimental challenge and infected vs. non-infected clams in the field exposure) allowed to detect spots shared by the two treatments of each comparison; however, treatment exclusive spots were only found in the comparison corresponding to the experimental challenge (Table VIII.2). The 25 treatment exclusive spots (10 of challenged clams and 15 of non-challenged ones) were excised from the stained gels, sequenced and annotated; twelve of them were found to correspond to known proteins (Table VIII.3).

Table VIII.1. Comparison of protein expression profiling between plasma pools from the same treatment in the two experiments: experimental challenge and field infection. For each treatment, diagonal cells show the number of spots shared by gel replicates from each pool. The number of spots shared between pairs of pools is shown in cells below the diagonal, and the percentage of similitude between pairs of pools is shown in cells above the diagonal.

Experiment	Treatment	# Pool	# Pool		
		# Pool			
Experimental Challenge	Challenged		**1**	**2**	
		1	267	62.6%	
		2	143	190	
	Non-challenged		**1**	**2**	
		1	303	70.4%	
		2	234	362	
Field Exposure	Infected		**1**	**2**	**3**
		1	102	64.0%	61.4%
		2	72	123	65.4%
		3	93	106	201
	Non-infected		**1**	**2**	**3**
		1	116	57.8%	57.3%
		2	63	102	62.6%
		3	80	83	163

Table VIII.2. Summary of information on spots in gels produced through 2DE with samples of proteins of *Ruditapes philippinarum* plasma, including the average number of spots in the replicate gels of each treatment, the number of spots in the master gel of each treatment, and the number of exclusive and shared spots in two pair comparisons between treatments: clams challenged with *P. olseni* zoospores vs. control clams in the experimental challenge and clams infected with *P. olseni* vs. non infected clams in the field exposure.

				Comparisons between treatments	
	Treatment	**Average no. spots in replicate gels**	**No. spots in master gels**	**No. exclusive spots**	**No. shared spots**
Experimental challenge	**Challenged**	360	143	10	105
	Non-challenged	418	234	15	
Field exposure	**Infected**	160	69	0	46
	Non-infected	189	54	0	

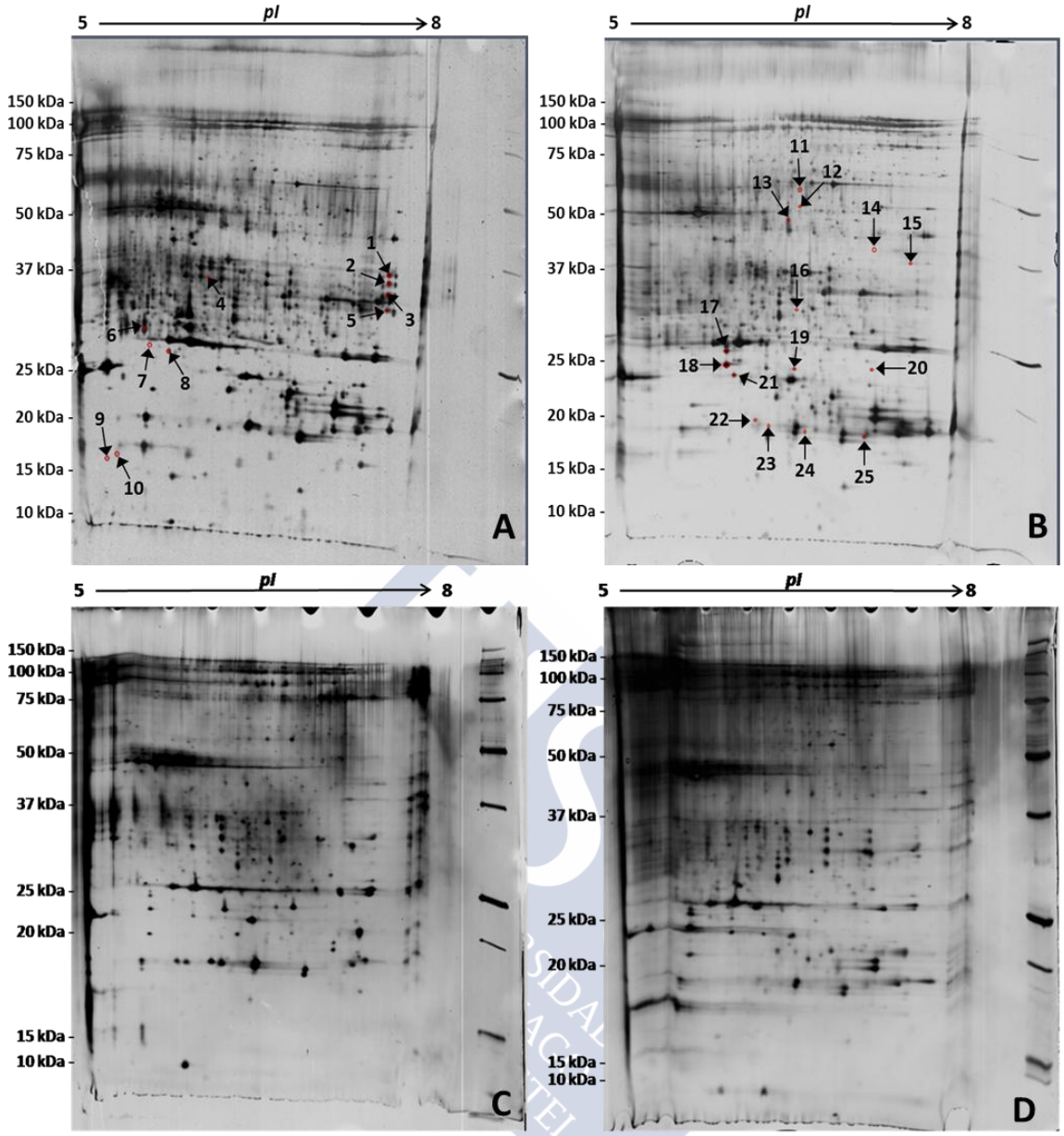

Fig. VIII.1. Representative 2-D gels pattern of proteins of *Ruditapes philippinarum* plasma corresponding to the four treatments. A. Clams challenged with *Perkinsus olseni* zoospores. B: Control clams in the experimental challenge. C: clams infected with *P. olseni* in field exposure. D: Non-infected clams in field exposure. Arrows show exclusive spots of each treatment.

Table VIII.3. Information on peptide sequence and protein annotation corresponding to the 25 spots excised from gels. Treatment: clams *Ruditapes philippinarum* challenged with *Perkinsus olseni* zoospores vs. control clams in the experimental challenge. Mw obs/theo: the molecular weight observed in gels and the theoretical one, respectively. pI obs/theo: the isoelectric point value observed in gels and the theoretical one, respectively. Match/(% Cov): number of amino acids of the protein sequence that corresponds to matched peptides and percentage. Score: score of the match of peptides with proteins in database after BLAST searching; the higher the value of the score, the better the peptide match. E-value: expected value, which describes the number of hits expected to see by chance when searching a database of a particular size; the lower the E-value, the better the peptide match. Sequence: only the best match sequence is shown in the table. Protein identification: protein name of identified peptides in the database. Accession number: accession number of proteins in the database.

Code	Treatment	Mw obs/theo	pI obs/theo	Match/ (%Cov)	Score	E-value	Sequence	Protein identification	Accession number
1	Challenged	50.2/47.56	7.9/8.50	8/9 (88%)	63.8	0.027	ELTCAVCSM	Hypothetical protein CGI_10018187 [*Crassostrea gigas*]	EKC36599.1
2	Challenged	48.8/76.19	7.9/9.82	10/13 (76%)	28.6	0.075	PKDKHKISIDPSG	Pin2-interacting protein X1 [*Crassostrea gigas*]	EKC29730.1
3	Challenged	47.4/37.66	7.9/6.26	8/10 (80%)	32.9	0.002	LCHDHRSTWY	Hypothetical protein CGI_10016495 [*Crassostrea gigas*]	EKC25986.1
7	Challenged	39.7/180	7.9/8.71	10/10 (100%)	45.4	0.003	VVNTMSGVII	Lysine-specific demethylase 3B [*Crassostrea gigas*]	EKC25077.1
8	Challenged	38.3/20.79	5.8/4.82	7/8 (87%)	35.4	2.00E-04	WWNEYVFR	Sarcoplasmic calcium binding protein [*Ruditapes philippinarum*]	BAA75223.1
9	Challenged	18.9/17.4	5.1/4.78	11/13 (83%)	42.6	4.00E-06	DSDINNDANCAVR	C-type lysozyme 2 [*Ruditapes philippinarum*]	AGO06639.1
11	Non-challenged	80.6/54.53	6.6/9.69	8/8 (100%)	27.8	1.50E-01	VDYNMLKR	Hypothetical protein CGI_10006409 [*Crassostrea gigas*]	EKC20269.1
12	Non-challenged	76,3	6,6	-	-	-	DSTVGVDDTT	No identification	-
13	Non-challenged	66.2/31.75	6.5/5.81	13/16 (80%)	32.5	0.005	GVTASTCEKYIIPGVN	Nicotinate-nucleotide pyrophosphorylase [*Crassostrea gigas*]	EKC35141.1
14	Non-challenged	56.3/29.37	7.2/5.28	10/11 (90%)	29.1	0.044	NHNDVIEGSRK	PDZ and LIM domain protein 5 [*Crassostrea gigas*]	EKC25487.1
16	Non-challenged	45.1/33.07	6.6/9.58	10/10 (100%)	57.1	0.1	LSSLSGGFGG	Pedal peptide 3 precursor [*Aplysia californica*]	NP_001191625.1
17	Non-challenged	37/66.85	6.0/6.55	9/12 (75%)	29.1	0.001	PVTATTTPVAMT	Lactadherin [*Crassostrea gigas*]	EKC26409.1
18	Non-challenged	33.6/34.25	6.0/4.80	10/12 (83%)	32	1.00E-04	IVMPAKNMDKGD	4-coumarate--CoA ligase-like 7 [*Crassostrea gigas*]	EKC22313.1
19	Non-challenged	36.2/108.64	6.6/5.99	11/15 (73%)	26.9	0.29	DMPDDPLEGDKKMAR	Hypothetical protein CGI_10011919 [*Crassostrea gigas*]	EKC23573.1
20	Non-challenged	30.8	7.2	-	-	-	YGSRVSGWASN	No identification	-
21	Non-challenged	32.3/22.01	6.0/5.20	7/7 (100%)	30.3	0.023	MEMKMER	Sialic acid-binding lectin [*Ruditapes philippinarum*]	ACU83226.1
22	Non-challenged	23.8/17.95	6.1/9.24	9/9 (100%)	30.3	0.13	TITDKEMTT	Hypothetical protein CGI_10009397 [*Crassostrea gigas*]	EKC42249.1
23	Non-challenged	23.5/16.81	6.3/9.3	10/12 (83%)	29.1	0.001	NSVINNAGNGYD	Caprin-2 [*Crassostrea gigas*]	EKC35775.1
24	Non-challenged	22.5/29.82	6.6/5.86	6/6 (100%)	26.1	0.052	MHMFSR	Ficolin-2 [*Crassostrea gigas*]	EKC22997.1
25	Non-challenged	22.6/18.97	7.1/4.64	8/8 (100%)	28.6	0.042	PGDDTTMM	Hypothetical protein CGI_10020194 [*Crassostrea gigas*]	EKC23814.1

VIII.5. DISCUSSION

Results showed high variability in the protein profile of the Manila clam plasma. *P. olseni* seemed to induce a decrease in the plasma protein diversity, both in the experimental challenge with zoospores and in field infection, although the differences in the spot numbers in gels from challenged and infected clams compared to those of non-challenged and non-infected clams, respectively, were not significant. The significant differences in the number of spots in the gels between non infected clams (field exposure) and non-challenged clams (laboratory challenge) should be due to the different environmental conditions of the clams (different geographic origin and different handling in terms of laboratory stay). High individual variability is deduced from the fact that master gels included less than 50% of the spots occurring in the gels of their respective biological replicates; that was more marked for the field exposure experiment in which only a reduced percentage of spots occurring in gels from the different pools were shared by all the pools, that is became included in the master gels. There is scarce information on factors influencing protein diversity of mollusc plasma. Most studies focused on particular proteins occurring in the plasma rather than protein diversity. Castellanos-Martínez et al. (2014) were able to identify 123 well-resolved spots in 2DE gels of *Octopus vulgaris* plasma; the authors also observed high variation between replicate gels of each sample, which was explained by the plasma sample pretreatment required to get well-resolved protein pattern with 2DE; however they did not identify statistically differential expressed proteins of octopus plasma due to infection with the protist *Aggregata octopiana*. We did detect qualitative changes in Manila clam plasma protein profile attributable to challenge with *P. olseni* zoospores: 15 spots became undetected while 10 other spots appeared; on the contrary, no differentially expressed protein was detected when comparing infected vs. non-infected clams from long-term field exposure, which was likely due to the low number of spots included in the master gels of the field exposure treatments, i.e. the lower the number of spots in master gels that are being compared the lower the probability of finding exclusive spots in one of the master gels.

Twenty out of 25 excised spots were successfully sequenced; eighteen of them were annotated and 12 were found to correspond to known proteins. The scarcity of sequences in mollusc databases, even after sequencing the Pacific oyster *C. gigas* genome (Zhang et al., 2012), and the particular scarcity of sequences of plasma or other extracellular proteins in databases difficult protein identification in non-model species. In contrast, higher identification success was achieved with proteins of Manila clam haemocytes (Chapter VII). A lysozyme, C-type lysozyme 2, was the one of the identified proteins appearing in clam plasma after challenge with *P. olseni* zoospores. Lysozymes play a role in defence and digestion in bivalves due to its muramidase activity (Soudant et al., 2013). Lysozyme are well known antibacterial enzymes that

cleave the glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine of peptidoglycan, a major cell wall component of bacteria. The antifungal and antiprotozoal activities of lysozymes have been attributed to the hydrolysis of N-acetylglucosamine linkages in chitin that makes up the cell wall of fungus and some protozoa (Bierman et al., 1979; Itoh et al., 2007). Lysozymes of a wide range of molluscs have been identified in plasma and tissue samples but, while the antimicrobial activity of lysozymes has repeatedly been demonstrated *in vitro*, the contribution of lysozymes to host defences is only beginning to be unravelled *in vivo* (Takeshita et al., 2004, Itoh et al., 2007). Four types of lysozyme have been found in molluscs thus far: c-, g-, i- and phage-type lysozyme; in the case of clams *R. philippinarum*, i- and phage-type had been reported (Ding et al., 2014). C-type lysozyme was found in *Haliotis discus discus*; its gene was up-regulated after bacterial and virus challenge indicating that it is involved in abalone immune response (Bathige et al., 2013). Up to three different lysozymes were found in oysters *C. virginica*, all of them inhibit the growth of Gram positive and Gram negative bacteria (Xue et al., 2004; Itoh et al., 2007; Xue et al., 2010); furthermore, antiprotozoal activity of cv-lysozyme 1 of *C. virginica* against *P. marinus* was reported (La Peyre et al., 2004). Ordás et al. (2000) did not find differences in lysozyme concentration between *P. olseni* non-infected and heavily infected clams *R. decussatus;* however, other studies showed up-regulation of lysozyme after exposure against pathogens, such as in clams *R. philippinarum* exposed to *Vibrio tapetis* (Allam et al., 2000) or to viral, bacterial and fungal PAMPs (Ding et al., 2014) and in mussels *M. galloprovincialis* exposed to *Vibrio angillarum* and *Micrococcus lysodictikus* (Costa et al., 2009). In our case, the expression of lysozyme 8 days after exposure to *P. olseni* zoospores could support that lysozymes play a role in the immune response against protozoan infection.

Pin2 interactin protein X1 (PinX1), lysine specific demethylase 3B and sarcoplasmic calcium binding protein (SCP) were the other identified protein appearing in plasma after challenge with *P. olseni* zoospores. PinX1 is involved in telomerase regulation and chromosome segregation in mitosis, it may inhibit cell proliferation and act as tumour suppressor; overexpression of PinX1 or its telomerase inhibitory domain suppress telomerase activity, causes telomerase shortening, and induces cells into crisis, whereas depletion of PinX1 increases telomerase activity and elongates telomeres (Yuan et al., 2009). In yeast, PinX1 is involved in rRNA and small nucleolar RNA maturation but it is not in telomere elongation inhibition (Guglielmi and Werner, 2002). It is difficult to understand the role of PinX1 in the clam response against *P. olseni*, perhaps it is involved in the regulation of the cell proliferation linked to inflammation. Lysine specific demethylase 3B is a protein involved in fundamental processes of changes in chromatin structure in response to external and internal stress actions, and also regulates processes of cellular growth, differentiation and gene expression in a histone-code-dependent manner (Mikhaleva et al., 2011). Its detection in the plasma of clams challenged with *P. olseni* zoospores deserves further research to

assess a specific role in the clam response against this parasite. SCPs are members of the EF-hand calcium binding protein family; proteins of this family have been selected evolutionarily with a range of affinities, kinetics and capacities to intervene in cell-specific Ca^{+2} signals. The rise and fall of the cellular Ca^{+2} concentration or in localised areas within the cytoplasm play a key role in the activation of membrane conductance, secretion, contraction, regulation of enzymes and genes. A further major task for these proteins is their "activator" function since Ca^{+2} ions in many cases need "interpreters" to activate or modulate cellular responses (Hermann and Cox, 1995). In shrimps *Penaeus vannamei*, SCPs are down-regulated in gills of animals infected by the yellow-head virus; the resulting impairment of cytosolic Ca^{+2} binding could lead to activation of enzymes that could damage or destroy the yellow head virus infected cells according to Rattanarojpong et al. (2007). In contrast to the shrimp case, SCP appeared up-regulated in clams challenged with *P. olseni* zoospores. This protein was also proposed as biomarker of stress levels in farmed *Penaeus monodon* haemolymph due to the changes suffered in protein expression during environmental stress (Tassanakajon et al., 2013). The modulation of these proteins could be an important key of the immune response of invertebrates to infections and of adaptation to stress conditions.

Three lectins became undetected in clam plasma after challenge with *P. olseni* zoopores, lactadherin, sialic acid binding lectin (SABL) and ficolin-2. Lactadherin is a glycoprotein which binds the lipid phosphatidylserine (PS) in an esterospecific and calcium-independent manner. It is secreted by a high number of cells in vertebrates (Otzen et al., 2012) and its gene has been detected in *C. gigas* genome (Zhang et al., 2012). Its involvement in protection against human infections is well-known (Newburg et al., 1998; Liu and Newburg, 2013). This protein is also involved in binding apoptotic cells by recognizing aminophospholipids such as PS to link apoptotic cells to phagocytes. Phagocytes engulf apoptotic cells rapidly to prevent the release of potentially noxious or immunogenic intracellular materials from the dying cells, thereby preserving the integrity and function of the surrounding tissue (Hanayama et al., 2002). SABL is a member of the immunoglobulin-like lectins family which promotes cell-cell interactions and regulates the functions of cells in the innate and adaptative immune system through glycan recognition. SABL acts as a pattern recognition receptor involved in the recognition of Gram-negative bacteria, it induces bacterial agglutination and activates phagocytes through binding sialic acid on foreign cells (opsonin activity) (Li et al., 2011). SABL appeared over-expressed in clams *R. philippinarum* infected with *P. olseni* compared to non-infected clams (Adhya et al., 2010) and it was up-regulated after challenge with pathogenic bacteria (Adhya et al., 2010; Li et al., 2011). Ficolins, a lectin family composed of mainly fibrinogen and collagen-like domains, were previously identified in other bivalves as *C. gigas*, *Saccostrea glomerata* and *Mya arenaria*, (Gueguen et al., 2003, Gagnaire et al., 2007, Green et al., 2009, Araya et al., 2010). As for other lectins, ficolins might act as a

phagocytic receptor for microorganism recognition (Gueguen et al., 2003). Lectins are highly expressed by bivalves and different lectins families have been identified, C-type lectins, galectins and sialic acid binding lectins. Lectins can be free in serum or bound to various other tissues or cells (Soudant et al., 2013). Previous studies had shown that clams *R. philippinarum* produce different lectin types, which are over-expressed after challenge with *P. olseni* and pathogenic bacteria (Kang et al., 2006, Kim et al., 2008a, Kim et al., 2008b; Moreira et al., 2012); some of them have been shown to bind *P. olseni* cells (Bulgakov et al., 2004). Considering the relevant role of lectins in the Manila clam response against *P. olseni*, the depletion of the three lectin types in the clam plasma eight days after challenge with *P. olseni* zoospores could be due to the binding of the lectins to *P. olseni* or to haemocytes; additionally, modulation of lectin expression varies in a time-dependent manner after challenge with *P. olseni* (Li et al., 2011).

Regarding the other identified plasma proteins that became undetected after challenge with *P. olseni* zoospores, the nicotinate-nucleotide pyrophosphorylase (NNP), also called quinolinate phosphoribosyl transferase, is a glycolsyltransferase involved in *de novo* NAD biosynthetic pathway (Bhatia and Calvo, 1996). NAD^+ activity is implicated in various cellular functions such as anti-oxidative activity, calcium homeostasis, gene expression and apoptosis; in fact, NNP interacts with active-caspase-3 to prevent spontaneous cell death through inhibition of overproduction of active-caspase-3 (Ishidoh et al., 2010). PDZ and LIM domain protein 5 (PDLIM5) is involved in zinc ion binding. LIM domains coordinate one or more zinc atoms, it consists of two zinc-binding motifs that resemble GATA-like zinc finger domains (Gamsjaeger et al., 2007). The proteins with a PDZ/LIM domain are involved in important but diverse biological roles such as cytoskeleton organisation, cell lineage specification, organ development and oncogenesis. Functionally, all PDZ and LIM domains share and important trait, these proteins can associate with and/or influence the actin cytoskeleton (Velthuis and Bagowski, 2007). Actin is a key protein in immune response for molluscs' haemocytes because is involved in phagocytosis, cell signalling, cell motility and vesicle and organelle movement (Chen et al., 2011). Interestingly, actin was down-regulated in haemocytes of clams challenged with *P. olseni* zoospores (chapter VII). The lack of detection of spots corresponding to PDLIM5 in plasma and actin in haemocytes after challenge with *P. olseni* zoospores could suggest a parasite induced alteration of host immune reaction. The pedal peptide 3 precursor is a neuropeptide found in a *Aplisia californica* ganglia EST library (Moroz et al., 2006); thus, *P. olseni* could interfere with host neurotransmission but its function is not well understood yet. The 4-coumarate CoA ligase like 7 is typical from plants. The protein is involved in the phenyl propanoid pathway that is exclusive from plants (Shin et al., 2010); its gene was detected in the genome of the Pacific oyster *C. gigas* (Zhang et al., 2012) but its role in molluscs needs further study. Caprin-2 (cytoplasmic activation/proliferation-associated protein 2) belongs to a family of cytoplasmic

proteins highly conserved in vertebrates; it is involved in cell proliferation and cell differentiation and is required for normal progression through the G1-S phase of the cell cycle (Ding et al., 2008; Sabile et al., 2013). Its role in clam-*P.olseni* interaction remains unknown.

In conclusion, twelve proteins occurring in Manila clam plasma were found to be modulated after challenging clams with *P. olseni* zoospores, while no plasma protein was unequivocally found to be modulated by longer term *P. olseni* infection. Some of the identified proteins have a well-known role in clam immune response against the parasite and the others deserve further study to better understand Manila clam-*P. olseni* interaction.

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IX. GENERAL DISCUSSION

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Perkinsosis of clams, the infection of clams with *P. olseni*, is a serious disease worldwide. The parasite (*P. olseni*) and the host (*R. philippinarum*) are the subject of this study. This dissertation includes experiments addressing the variability of the parasite and the modulation of the host immune response by the infection. The results have been thoroughly discussed in the previous chapters, thus this general discussion aims to provide an integrative scope trying to minimise repetition.

Variability of the parasite.

P. olseni clones deriving from 4 locations of the Spanish coast were used to analyse variability by two approaches, analysis of population genetic structure, using microsatellite markers, and proteomic comparison, considering cellular and released proteins. Population genetic analysis showed low genetic variation in *P. olseni* Spanish populations compared with *P. olseni* clones from Japan and New Zealand. Very likely, the parasite was introduced into Europe with the importation of infected clams from the Pacific area, and then into the Spanish coast (ca. 1980s), thus the low microsatellite polymorphism could be reflecting founder effects in the recent evolutionary history of *P. olseni.* Consistently, the comparison of cellular protein profiles also showed low variability among clones from the Spanish coasts. On the contrary, a substantial allelic richness in every microsatellite loci of *P. marinus* was reported along the Gulf and Atlantic coast of the USA, in populations where the parasite occur since 1940s or earlier and even in populations where parasite was established recently (Thompson et al. 2014a). Remarkably, higher variability was observed in the protein profile of ECPs among the clones from the Spanish coasts; these higher variability of the released proteins could result in a better adaptation to the environment in each location and could be associated with differences in virulence or in resistance to host defences between *P. olseni* clones from different locations. ECPs can be released due to very different circumstances and the results could be highly variable among individuals (La Peyre et al., 2010).

Geographic distance was not correlated with similitude between *P. olseni* clones from the four Spanish locations in any experimental approach (population genetic structure, cellular protein profile and extracellular protein profile). It suggests that the dispersal of the parasite due to ocean currents has been less important than man-driven movements of infected clams in shaping the population genetic structure of the parasite.

The relative distance between three *Perkinsus* spp., *P. olseni*, *P. marinus* and *P. chesapeaki*, deduced from the proteomic approach is consistent with the relative genetic distance deduced from the comparison of their rDNA gene sequences (Moss et al., 2008; da Silva et al., 2014). Funding limitations avoid providing a wide scope of identified proteins because identification of exclusive rather than shared proteins has been emphasised in every experiment. High constitutive expression of

antioxidant/detoxifying proteins that could be involved in defence and prevention of oxidative damage produced by host immune response was detected in *Perkinsus* spp.; peroxiredoxins (Prx) II and V were identified in *Perkinsus olseni*, both in cells and ECPs, as well as in *P. chesapeaki* cells. Other proteins of the antioxidant/detoxifying group, as superoxide dismutase and glutathione S-transferase, were found in *P. olseni* and *P. chesapeaki* respectively. Other proteins, vacuolar ATPase subunit b and g, malate dehydrogenase and receptor for activated C kinase (RACK) were shared by *P. marinus, P. olseni* and *P. chesapeaki*. RACK seems to have an important role in cell invasion of the parasite modulating the Ca^{+2} concentration in the host (Sartorello et al., 2009). Regarding proteins identified for the analysis of *P. olseni* variability through the Spanish coast, the fact that heat shock proteins HSP 90 and HSP 60, cathepsin-β and pepsin A were found exclusively in clons from the Ría de Arousa could involve an adaptive advantage and it suggests higher pathogenicity of the *P. olseni* from this location, which should be further studied.

Response of the host

The modulation of protein profiling of haemocytes and plasma of the Manila clam by infection with *P. olseni* was addressed considering short and long-term effects. This study contributes as a first step to know the proteome of haemocytes and plasma of the clam and has allowed identifying proteins related with the clam immune response. Challenging clams with parasite zoospores provoked appearance of HSP70-12B and integrin alpha-PS3, whereas cytochrome C oxidase, actin and ankyrin-3 became undetected in haemocytes; in the plasma, that challenge caused appearance of a C-type lysozyme, sarcoplasmic calcium binding protein, lysine-specific demethylase 3B and pin2-interacting protein X1, whereas three lectins (lactadherin, sialic acid binding and ficolin-2), nicotinate-nucleotide pyrophosphorylase, PDZ and LIM domain protein 5, pedal peptide 3 precursor, 4-coumarate-CoA ligase-like 7 and caprin-2 became undetected.

After long-term field exposure to *P. olseni*, two proteins were found to mark differences between clams with advanced infection and non-infected clams: transcriptional regulator ATRX was exclusively found in clams in which infection progressed, whereas Rho GTPase-activating protein 6 was exclusively detected in clams in which infection was not detected (in spite of long-term exposure to the parasite), i.e. in potentially resistant clams. Thus, Rho GTPase-activating protein 6 could hypothetically be a marker of resistance against *P. olseni* infection, which deserves further assessment. Availability of markers of resistance would allow developing efficient marker-assisted selective breeding programmes to produce Manila clam strains resistant against *P. olseni* infection.

X. CONCLUSIONS

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I. Evidence of sexual reproduction in the life cycle of *P. olseni* was found.

II. Low polymorphism in *P. olseni* populations through the Spanish coast was found, much lower than in populations from Japan and New Zealand, which suggests founder effects due to historically-recent introduction of the parasite into Europe through importation of clams from Pacific areas.

III. Proteomic approach also showed high similitude among *P. olseni* clones through the Spanish coast.

IV. Identification of HSP 60, cathepsin-β and pepsin A exclusively in the extracellular products of *P. olseni* clones from Ría de Arousa suggests differences in virulence and/or in resistance to host attack with regard to clones from the other regions, which should be further studied.

V. High constitutive expression of antioxidant/detoxifying proteins linked to evasion of host defence was observed in cells and extracellular products of *Perkinsus olseni* as well as in cells of *Perkinsus marinus* and *Perkinsus chesapeaki*. Protein profile comparison among three *Perkinsus* species showed higher similitude between *P. marinus* and *P. olseni* than *P. chesapeaki* with them.

VI. Seven Manila clam haemocyte proteins of which expression was markedly affected by *P. olseni* were identified. Among them, Rho GTPase-activating protein 6 is a candidate for further study to assess its validity as marker of resistance against *P. olseni* infection.

VII. The occurrence of twelve identified proteins in Manila clam plasma was dependent on infection with *P. olseni*. Among them, three lectins became undetected while a C-type lysozyme appeared after challenging clams with *P. olseni* zoospores.

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XII. ANNEX I:
SUPPLEMENTARY MATHERIAL AND METHODS

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Sequencing of proteins by LC-MS/MS was performed as follows: samples were in-gel digested with trypsin (Promega, Madison, WI) using a Digest MSPro (Intavis, Koeln, Germany). Briefly, gel slices were washed with water and 20 mM ammonium bicarbonate pH 7.8, reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide and digested with trypsin for 16 h at 37 ºC. Tryptic peptides were extracted with acetonitrile/water 0.25% TFA. Extracts were evaporated to dryness by vacuum and redisolved in 5 µL MeOH/H2O 2/1 0.1% TFA. MALDI-TOF spectra for each sample were obtained to ensure that the peptide contents were sufficient for successful LCMS analysis. Samples with detectable peptide concentrations were analysed by LC-MS/MS using a high resolution LTQ/Orbitrap mass spectrometer equipped with a microESI ion source (ThermoFisher, San Jose, CA). Each extract was diluted up to 40 µL with 1% formic acid. Samples were loaded in a chromatographic system consisting of a C18 preconcentration cartridge (Agilent Technologies, Santa Clara, CA) connected to a 10 cm long, 75 µm i.d. Vydac C18 column (Vydac, IL, USA). The separation was done at 0.4 µL/min in a 30 min acetonitrile gradient from 3 to 40% (solvent A: 0.1% formic acid, solvent B: acetonitrile 0.1% formic acid). The HPLC system was composed by an Agilent 1200 capillary nano pump, binary pump, a thermostated microinjector and a micro switch valve. The Orbitrap instrument was operated in the positive ion mode with a spray voltage of 2 kV. The scan range of each full MS was m/z 400-2000. The spectrometric analysis was performed in an automatic dependent mode, acquiring a full scan and 5 MS/MS of the most abundant signals. Both MS and dependent MSMS scans were performed in the FT mode to maximise the mass resolution and precision of the MS spectra. A dynamic exclusion was set to 1 to avoid the redundant selection of precursor ions.

MS/MS spectra were used for searching protein sequences using SEQUEST (Proteome Discoverer, ThermoFisher, San Jose, CA) with the following parameters: peptide mass tolerance 10 ppm, fragment tolerance 100 ppm, enzyme set as trypsin and allowance up to two missed cleavages, static modification was cysteine carbamidomethylated (+57 Da) and dynamic modification was methionine oxidation (+16 Da). The Uniprot database (taxonomy: Alveolata) was used for searching. Identifications were filtered with Sf > 0.6, P(pep) < 0.005 and were manually validated. The chromatograms that gave negative identification with database search were reanalysed using the PEAKS *de novo* search engine tool (PEAKS Studio 5.2, Bioinformatics Solutions Inc., Waterloo, ON, Canada). The *de novo* search parameters were: mass tolerance 10 ppm for precursor ions and 100ppm for fragment ions. *De novo* sequences with an ALC score higher than 70% were sent to the Basic Local Alignment Search Tool (BLAST) for protein identification. Uniprot database (taxonomy Alvelolata) and SwissProt database (taxonomy: mammals + human) were used in BLAST. The mammals+human database was used in order to detect sequence tags related to probable keratin and trypsin contaminations. These sequence tags were rejected.

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XIII. RESUMEN

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El cultivo de almejas es una actividad que crece cada año a nivel mundial. La especie de almeja de mayor producción en el mundo es la almeja japonesa *Ruditapes philippinarum*. El país con mayor producción de esta especie es China, seguido por Italia, Corea del Sur, EE. UU. y España. La almeja japonesa se introdujo en Europa durante las décadas de los 70 y 80 del pasado siglo y se expandió rápidamente, naturalizándose en varios países europeos. *R. philippinarum* está bien adaptada y crece más rápido que la especie nativa *Ruditapes decussatus*, por lo que se ha convertido en la almeja de mayor producción en Europa. La producción de almejas venéridas en Galicia, considerando marisqueo y cultivo, es un recurso socio-económico muy importante. También en Galicia el cultivo de almeja japonesa se ha incrementado en los últimos años, convirtiéndose en la especie de almeja cultivada con producción más alta.

La infección por *Perkinsus olseni* es una de las enfermedades más serias que afectan a las almejas. Este parásito está muy ampliamente distribuido por el mundo, afectando a una larga lista de moluscos en los cinco continentes. La infección por *P. olseni* se ha asociado a episodios de mortandad de *R. decussatus* y *R. philippinarum* en áreas del Sur de Europa así como de *R. philippinarum* en varios países asiáticos. Dos especies del género *Perkinsus*, *P. olseni* y *P. marinus*, están incluidas en la lista de enfermedades de declaración obligatoria de la Organización Mundial de Sanidad Animal, lo que indica el interés internacional por frenar su expansión. El conocimiento sobre *P. olseni* es escaso si se compara con el de *P. marinus*; ésta provoca mortandades masivas de ostras *Crassostrea virginica* en EE. UU. La transcendencia económica de la perkinsosis de las almejas justifica la investigación encaminada a conocerla mejor, para encontrar vías por las que minimizar sus efectos. Con esta intención se desarrolló este estudio, para conocer (1) la variabilidad de *P. olseni* en la costa española, con énfasis en la variabilidad de su virulencia, y (2) la modulación de la expresión proteínica de la almeja japonesa debida a la infección por *P. olseni*, con énfasis en la búsqueda de marcadores proteínicos de resistencia a esta infección. El estudio de la variabilidad de *P. olseni* se abordó analizando la estructura genética poblacional así como comparando el proteoma de clones de *P. olseni* derivados de varias regiones repartidas por el litoral español. Además se comparó el proteoma de *P. olseni* con los de otras dos especies de *Perkinsus* spp., *P. marinus* y *P. chesapeaki*, para así ampliar la perspectiva de la variabilidad del parásito. El análisis de la modulación de la expresión proteínica de la almeja japonesa debida a la infección por *P. olseni* se enfocó en los hemocitos y la hemolinfa, con objeto de concentrar la atención en la modulación de la respuesta inmunitaria de la almeja por el parásito; se consideró el efecto en la almeja de la exposición al parásito a corto plazo así como tras un periodo muy prolongado.

Los objetivos concretos de este estudio fueron los siguientes:

1. Evaluación de la variabilidad genética de *P. olseni* en el litoral español usando marcadores microsatélite.

2. Evaluación de la variabilidad de los perfiles proteínicos celulares y extracelulares de *P. olseni* en el litoral español.

3. Comparación de los perfiles proteínicos celulares de tres especies del género *Perkinsus*: *P. olseni*, *P. marinus* y *P. chesapeaki.*

4. Identificación de proteínas de hemocitos y plasma de la almeja *R. philippinarum* cuya expresión está modulada por la infección por *P. olseni*.

5. Identificación de proteínas marcadoras de resistencia a la infección por *P. olseni* en la almeja *R. philippinarum*.

Capítulo 1. Evaluación de la variabilidad genética de *Perkinsus olseni* entre regiones del litoral español mediante el uso de marcadores microsatélite .

Hay escasa información sobre una serie de parámetros poblacionales de *P. olseni* relevantes, como variabilidad genética, patrones de dispersión, estructura genética poblacional y estrategias reproductivas. Se sabe que el parásito se propaga por amplificación clonal, tanto dentro como fuera del hospedador, y esto debería determinar la estructura genética poblacional, aunque la existencia de reproducción sexual ha sido postulada previamente sin que todavía se haya determinado en qué punto del ciclo de vida tiene lugar. Conocer bien todos estos aspectos es muy importante para la gestión de cualquier enfermedad parasitaria. Para conseguir información en este contexto, se desarrolló un estudio de la variabilidad genética de *P. olseni* considerando las tres regiones de mayor producción de almejas en España, usando marcadores microsatélite. En concreto se analizaron muestras de dos rías gallegas (Arousa y Pontevedra), Andalucía occidental (río Carreras, Huelva) y el Delta del Ebro. Además, como referencia se utilizaron cultivos clonales de *P. olseni* derivados de parásitos aislados de moluscos de Japón y Nueva Zelanda. Para garantizar la disponibilidad de un número suficiente de células idénticas del parásito en cada réplica de cada región, se utilizaron cultivos clonales producidos *in vitro*.

Un total de 130 cultivos clonales de *P. olseni,* provenientes de las seis regiones mencionadas, fueron genotipados con 12 microsatélites diseñados expresamente para el estudio. El análisis de la variación de los 12 microsatélites de las diferentes regiones de la Península Ibérica mostró un déficit de heterozigotos muy marcado con respecto a

las expectativas relativas al equilibrio Hardy-Weinberg. Tanto el polimorfismo como el número de alelos fueron bajos, observándose una estructura genética moderada entre poblaciones y una elevada estructura genética entre parásitos de almejas diferentes. A pesar del marcado déficit de heterozigotos, su existencia sugiere que el parásito tiene una fase diploide en su ciclo de vida, confirmando la reproducción sexual. Este déficit de heterozigotos es explicado por el efecto Wahlund (la reducción de la heterozigosis en una población es causada por la existencia de subpoblaciones). Ello sugiere que las poblaciones del parásito están formadas por infrapoblaciones constituidas por los parásitos presentes en cada uno de los hospedadores infectados. Las distancias genéticas entre clones de diferentes almejas del litoral español fue relativamente alta, independientemente de la región de origen, y las infrapoblaciones mostraron diferenciación genotípica significativa. Estos resultados sugieren endogamia dentro de las infrapoblaciones, con una frecuente recombinación sexual y no una estructura poblacional únicamente clonal.

Las muestras procedentes del área Asia-Pacífico eran muy diferentes de las del litoral español y mostraron mayor variabilidad. Este hallazgo es coherente con la hipótesis de que *P. olseni* fue introducido en la Península Ibérica debido a importaciones de almeja japonesa infectada con el parásito, con el consiguiente efecto fundador que explicaría una pérdida severa de variación genética en las poblaciones españolas del parásito. Por otro lado, se observó una falta de correlación entre distancia génica y geográfica en el litoral español, lo que sugiere que la dispersión del parásito debida a las corrientes oceánicas tiene una menor importancia que la dispersión ocasionada por el movimiento de lotes comerciales de almejas.

Capítulo 2. Variabilidad del proteoma celular de *Perkinsus olseni* entre regiones de la costa española.

En este capítulo se abordó la comparación del proteoma celular de *P. olseni* de las regiones del litoral español incluidas en el capítulo 1, con el fin de evaluar la variabilidad del parásito en el litoral español con metodología diferente. Para ello se aislaron parásitos *P. olseni* de cuatro almejas *R. decussatus* de la ría de Arousa, cuatro de la ría de Pontevedra, cuatro del río Carreras y cuatro almejas *R. philippinarum* del Delta del Ebro. Con los parásitos aislados de cada almeja se iniciaron cultivos *in vitro* (uno por almeja), que a su vez se clonaron. Un clon de *P. olseni* derivado de cada una de las almejas, es decir cuatro clones de cada origen geográfico (16 clones en total) se cultivaron *in vitro* para obtener un número de células idénticas suficientes de cada clon para análisis proteómico. Se aislaron las proteínas celulares de muestras de cada clon y se separaron mediante electroforesis bidimensional, obteniéndose marcas proteínicas (*spots*) en geles de poliacrilamida. Los geles se tiñeron con plata para visualizar las marcas proteínicas y se digitalizaron. Se produjeron cuatro geles de cada clon,

descartándose el peor de ellos, de forma que se utilizaron tres geles (réplicas analíticas) de cada clon. Los geles digitalizados se analizaron con el programa informático PD Quest V.7.4.0.

Se obtuvieron más de 600 marcas por gel, de las que 141 eran comunes a todos los clones de las 4 regiones, mientras que 39 marcas eran exclusivas de la ría de Arousa, 57 exclusivas de la ría de Pontevedra, 37 del río Carreras y 47 del Delta del Ebro. La comparación entre regiones por pares mostró porcentajes de similitud entre 55 y 72%; Las regiones atlánticas mostraron más similitud entre ellas que la estimada entre el Delta del Ebro y las regiones atlánticas, aunque el porcentaje de similitud entre las regiones atlánticas no se correlacionaba con la distancia geográfica. Debido a una insuficiencia presupuestaria, sólo una parte de las marcas proteínicas pudieron procesarse para su secuenciación por espectrometría de masas y posterior identificación en bases de datos. En concreto 19 marcas comunes a todos los clones de las 4 regiones y 35 marcas exclusivas de alguna de las regiones (7 de la ría de Arousa, 11 de la ría de Pontevedra, 8 del río Carreras y 9 del Delta del Ebro) se procesaron. Del total de las 54 marcas procesadas, se pudieron secuenciar 22 e identificar 19 proteínas (13 proteínas comunes a todas las regiones y 6 específicas de clones de una región). La mayor parte de las proteínas identificadas correspondieron a proteínas implicadas en procesos metabólicos, pero entre ellas cabe destacar la enolasa-2. Esta proteína presenta un pentapéptido estrictamente conservado en plantas, ya encontrado previamente en *P. marinus*, lo que es coherente con la filogenia del parásito, con un ancestro común con dinoflagelados. Se constató también una elevada expresión constitutiva de proteínas antioxidandes como la superóxido dismutasa y las peroxiredoxinas II y V, que podrían estar implicadas en la neutralización de especies reactivas tóxicas de oxígeno liberadas por los hemocitos del hospedador como parte de la respuesta inmunitaria de éste. En el caso de las proteínas exclusivas de cada población, se identificaron la galactokinasa y una proteína de choque térmico 90 (HSP 90) en clones de la Ría de Arousa, la piruvato kinasa en la Ría de Pontevedra, la malato deshidrogenasa en el Delta del Ebro y la glyceraldehido 3-fosfato deshidrogenasa en el río Carreras. Estas proteínas del grupo de las exclusivas de una zona están todas implicadas en procesos del ciclo de Krebs excepto la HSP90; es reseñable que ninguna de las proteínas exclusivas identificadas estaba directamente implicada en procesos de virulencia o toxicidad, por lo que no se detectaron diferencias asociadas a virulencia entre regiones.

Capítulo 3. Variabilidad de los perfiles de expresión proteínica en los productos extracelulares entre regiones de la costa española.

En este capítulo se abordó la comparación de los perfiles proteínicos de los productos extracelulares de *P. olseni* de las regiones del litoral español mencionadas

en los capítulos previos. Se había constatado en varias especies del género *Perkinsus*, la liberación de enzimas hidrolíticas que degradan los tejidos del hospedador, por lo que los productos extracelulares del parásito incluyen factores de virulencia. El estudio incluido en este capítulo pretendió evaluar la variabilidad del perfil proteínico de los productos extracelulares del parásito en el litoral español, con énfasis en las diferencias asociadas a la virulencia. Como en el capítulo anterior, se utilizaron cuatro clones de cada origen geográfico (16 clones en total). Cada clon derivaba de un hospedador diferente. Los clones se cultivaron *in vitro* y las proteínas liberadas por el parásito al medio de cultivo se aislaron y se separaron mediante electroforesis bidimensional en geles de poliacrilamida. El tratamiento de los geles fue similar al descrito en el capítulo previo.

Se visualizaron entre 118 y 144 marcas en los geles, de las que 23 eran comunes a todos los clones de las 4 regiones, mientras que 5 eran específicas de la ría de Arousa, 14 de la ría de Pontevedra, 21 del Delta del Ebro y 4 del río Carreras. La comparación entre regiones por pares mostró porcentajes de similitud entre 44 y 72%, siendo la ría de Arousa la región más dispar. La insuficiencia presupuestaria sólo permitió procesar 34 marcas para su secuenciación por espectrometría de masas y posterior identificación en bases de datos, 11 comunes a los clones de todas las regiones y 23 específicas de una región (4 de la ría de Arousa, 7 de la ría de Pontevedra, 10 del Delta del Ebro y 2 del río Carreras). De las 34 marcas procesadas, se consiguió secuenciar 17 e identificar 10 proteínas (5 proteínas comunes a clones de todas las regiones y 6 específicas de clones de una región)

Entre las proteínas comunes a clones de todas las regiones, una de las identificadas fue la subunidad β del receptor de reconocimiento de señales, que forma parte de un complejo implicado en el transporte de proteínas a través de las membranas celulares. .También se identificó la cathepsina β, una peptidasa cisteínica, siendo ésta la primera vez que se identifica una proteasa cisteínica en los productos extracelulares de *P. olseni*. Se ha constatado el papel relevante de este grupo de peptidasas en otros protistas parásitos, contribuyendo a la invasión y degradación de los tejidos del hospedador. En cuanto a las proteínas exclusivas de clones de una región, tres de ellas correspondían a los clones de la ría de Arousa, pepsina A, que permite al parásito la obtención de nutrientes y energía mediante la degradación de tejidos del hospedador a la par que facilita el progreso de la infección, fosfoserina aminotransferasa, que está implicada en la síntesis de L-serina, componente esencial de la síntesis proteica, y la proteína de choque térmico 60 (HSP60), relacionada con procesos de estrés celular, con papel esencial en la síntesis, transporte y plegado de las proteínas. Además, se identificó una peroxiredoxina correspondiente a una marca exclusiva de la ría de Pontevedra; la función de las peroxiredoxinas en la neutralización de especies reactivas tóxicas de oxígeno liberadas por los hemocitos del hospedador se ha mencionado en el capítulo previo. En suma, la variabilidad entre clones de *P. olseni*

de las cuatro regiones del litoral español en las proteínas extracelulares fue mayor que en las celulares; algunas de las diferencias detectadas entre regiones estaban asociadas a virulencia. Las diferencias en virulencia entre clones de regiones diferentes del litoral español deberían estudiarse más a fondo, dada su transcendencia para la gestión de la perkinsosis.

Capítulo 4. Comparación de los perfiles de expresión proteínica entre tres especies de *Perkinsus*, *P. olseni*, *P. marinus* y *P. chesapeaki*.

El análisis de la variabilidad del agente etiológico de la perkinsosis se completó a través de la comparación del proteoma celular de tres especies del género *Perkinsus*. Se partió de un cultivo clonal de cada especie, *P. marinus*, *P. olseni* y *P. chesapeaki*. Tras aislar las proteínas celulares, éstas se separaron mediante electroforesis bidimensional en geles de poliacrilamida (cuatro réplicas analíticas por especie). Los geles se tiñeron con Sypro Ruby, se digitalizaron y se compararon con el programa informático Proteomweaver 4.0. Más de 1600 marcas proteínicas se observaron en los geles de cada especie. Se constató que 213 marcas eran comunes a las tres especies, 310 eran compartidas por *P. marinus* y *P. chesapeaki*, 315 por *P. chesapeaki* y *P. olseni*, y 242 por *P. marinus* y *P. olseni*. Se encontraron marcas específicas de cada especie: 1161 de *P. chesapeaki*, 1124 de *P. olseni* y 825 de *P. marinus*. Un análisis de similitud mostró mayor proximidad entre las especies *P. marinus* y *P. olseni*, siendo *P. chesapeaki* la más alejada de las tres, lo que es coherente con resultados previos basados en la comparación de las secuencias del gen ribosómico y de la actina.

Se seleccionaron 14 marcas comunes a las tres especies y 14 marcas específicas de cada especie para su secuenciación por espectrometría de masas y su posterior identificación en bases de datos. Las 14 marcas comunes se separaron por triplicado (una marca de un gel de cada especie). De las 84 marcas procesadas, se logró la secuencia de 42 y 28 secuencias se identificaron con proteínas de las bases de datos del NCBI y SwissProt. Únicamente 6 proteínas comunes pudieron ser identificadas, las subunidades β y g de la ATPasa vacuolar, la malato deshidrogenasa, el receptor de la quinasa C activada, la pseudouridina sintasa, y la fosfato acetiltransferasa. Con respecto a las proteínas específicas, en *P. olseni* se identificó la formato deshidrogenasa, una subunidad del proteasoma, la triosafosfato isomerasa y la peroxiredoxina V; en *P. marinus* la fosfogliceto kinasa y la proteína 40S ribosomal S3 y en *P. chesapeaki* se identificó la glutatión S-transferasa, la peroxiredoxina II y la peroxiredoxina V (ésta en dos marcas). Las peroxiredoxinas y la glutation S-transferasa están implicadas en funciones de detoxificación y antioxidación, pudiendo actuar para neutralizar las especies reactivas tóxicas de oxígeno que genera el hospedador para luchar contra el avance de la infección. También el receptor de la quinasa C activada tiene un papel relevante en la regulación de Ca^{+2} en el hospedador; permitiría al

parásito modular procesos como la apoptosis, proliferación celular y disponibilidad de nutrientes. Las restantes proteínas identificadas regulan procesos vitales en el organismo como son el metabolismo, modificaciones post-transcripcionales, diferenciación celular y regulación del pH intracelular del parásito.

Capítulo 5. Expresión proteínica de los hemocitos de la almeja japonesa *Ruditapes philippinarum* en respuesta a la infección por *Perkinsus olseni*.

La hemolinfa es la sangre de los moluscos, en ella se distinguen dos componentes, las células (hemocitos) y el plasma. Los hemocitos están implicados en la reacción inmunitaria de la almeja a través de la fagocitosis o encapsulación de patógenos y su posterior destrucción y liberación de moléculas implicadas en la respuesta inmune. El plasma también contiene factores implicados en la respuesta inmunitaria, como lectinas, péptidos antimicrobianos, lisozimas o inhibidores de proteasas liberadas por parásitos, entre otros. Para conocer la respuesta inmunitaria de la almeja japonesa *R. philippinarum* ante la infección por *P. olseni*, se analizó la modulación del perfil de expresión proteínica de los hemocitos y del plasma de la almeja cuando ésta se enfrenta al parásito. En este capítulo se recoge el estudio correspondiente a los hemocitos y en el siguiente capítulo el correspondiente al plasma. Se plantearon dos situaciones experimentales, para conocer los efectos del enfrentamiento a corto plazo así como tras un enfrentamiento prolongado. En el análisis de los efectos a corto plazo se utilizaron almejas recogidas en un banco natural de Camariñas (A Coruña), donde nunca se había detectado la presencia de *P. olseni*. De ellas, 40 almejas se expusieron en vasos individuales a 10^6 zooesporas de *P. olseni* en agua de mar durante 24 horas, tras lo que se mantuvieron en un tanque durante 7 días; Otras 40 almejas se sometieron a las mismas condiciones pero sin añadir zooesporas a los vasos, para utilizarlas como control del experimento. Se extrajo hemolinfa de cada almeja, mezclándose la hemolinfa por grupos de 20 almejas, para producir así dos réplicas biológicas de almejas enfrentadas al parásito y otras dos de almejas no enfrentadas. Las muestras de hemolinfa se procesaron para separar los hemocitos del plasma y analizar la expresión proteínica, comparando la de los hemocitos de almejas enfrentadas al parásito con la de los hemocitos de almejas no enfrentadas.

En el análisis de la exposición prolongada al parásito, se recogieron almejas de tamaño comercial (al menos dos años de edad) en un banco natural afectado por *P. olseni*, con valores altos de intensidad de infección, localizado en Vilalonga (ría de Arousa). Se trataba por tanto de almejas expuestas de manera natural al parásito durante un tiempo prolongado. Se extrajo la hemolinfa de 200 almejas y se diagnosticó la intensidad de la perkinsosis de cada una de ellas mediante el método de incubación de branquia en caldo de tioglicolato; se seleccionó la hemolinfa de 42 almejas que

presentaban una infección avanzada (entre 3 y 5 en la escala de Mackin) así como la hemolinfa de otras 42 almejas en que no se detectó infección. La hemolinfa de las almejas seleccionadas se mezcló por grupos de 14 almejas, para producir así tres réplicas biológicas de almejas con infección avanzada y otras tres de almejas no infectadas. Las muestras de hemolinfa se procesaron para separar los hemocitos del plasma y analizar la expresión proteínica, comparando la de los hemocitos de almejas con infección avanzada con la de las almejas no infectadas.

En ambos planteamientos experimentales, las proteínas hemocitarias se aislaron y se separaron mediante electroforesis bidimensional en geles de poliacrilamida. Los geles se tiñeron con plata, se digitalizaron y se compararon (almejas expuestas frente a no expuestas en la exposición en laboratorio; almejas con infección avanzada frente a almejas no infectadas en la exposición natural prolongada) usando el programa informático PD Quest 8.4.0. Los geles mostraron un promedio de 570 marcas proteínicas en ambos planteamientos experimentales. No se detectaron diferencias significativas en el número de marcas por gel entre tratamientos. En el caso de la exposición a *P. olseni* en laboratorio, se han observaron tres marcas exclusivas en muestras de almejas expuestas y cinco exclusivas en las almejas no expuestas; en el caso de exposición prolongada en el medio natural, se detectaron dos marcas exclusivas en muestras de almejas infectadas y una en las no infectadas. Todas esas marcas se seleccionaron para su secuenciación por espectrometría de masas y posterior identificación en bases de datos; de ellas sólo siete pudieron ser identificadas.

Parece que la exposición a *P. olseni* en el laboratorio indujo la expresión de la proteína de choque térmico 70 (HSP 70) 12B y la integrina-α PS3 en los hemocitos de las almejas, pues ambas proteínas se identificaron en marcas exclusivas de almejas expuestas. La proteína HSP 70 12B está implicada en la respuesta inmune de los moluscos bivalvos; se ha constatado su sobreexpresión en varias especies de moluscos expuestas a patógenos. La integrina-α PS3 es una glicoproteína implicada en el proceso de encapsulación de patógenos por hemocitos, así como en la fagocitosis de células apoptóticas y de bacterias, en varios invertebrados. Por el contrario, la exposición al parásito en el laboratorio pudo haber inhibido la expresión de citocromo C oxidasa I, actina y ankyrina-3, puesto que se identificaron en marcas exclusivas de hemocitos de almejas no expuestas. La disminución de la expresión de actina parece una respuesta común de los invertebrados frente a la infección. Actina y Ankyrina están implicadas en procesos de movilidad celular, regulación del citoesqueleto e incluso de apoptosis, mientras que la citocromo C oxidasa I está implicada en la obtención de energía de la cadena de transferencia de electrones en la mitocondria.

En el caso de la exposición prolongada al parásito en el medio natural se identificó el regulador posttranscripcional ATRX, correspondiente a una marca

exclusiva de hemocitos de almejas no infectadas. Esta proteína está implicada en varios procesos celulares como, metilación del DNA, transcripción, ciclo celular y apoptosis. Su función en procesos de respuesta inmune no está bien dilucidada. La Rho GTPasa 6 se identificó en una marca exclusiva de hemocitos de almejas no infectadas, por tanto podría tratarse de una proteína cuya expresión se inhibe por la infección o bien de una proteína cuya presencia impide que la almeja se infecte por *P. olseni* o que la infección no progrese. Si éste fuese el caso, esta proteína podría constituir un marcador molecular de resistencia a la perkinsosis. Su función está relacionada con la organización de actomiosina, adhesión, y proliferación; aunque también forma parte de rutas de señalización usadas por receptores de antígenos que regulan la respuesta inmune. La enorme importancia de contar con un marcador molecular de resistencia a la perkinsosis para implementar programas de selección genética para producir estirpes de almeja resistentes a la perkinsosis, aconseja analizar a fondo si realmente la Rho GTPasa 6 es un marcador de resistencia al parásito.

Capítulo 6. Estudio de la expresión proteínica del plasma de almejas *Ruditapes philippinarum* en respuesta a la infección por *Perkinsus olseni*.

El planteamiento experimental es el expuesto en el capítulo previo. En este caso, las muestras de plasma se procesaron para aislar las proteínas y separarlas mediante electroforesis bidimensional en geles de poliacrilamida. Los geles se tiñeron con plata, se digitalizaron y se compararon. No se detectaron diferencias significativas en el número de marcas proteínicas entre muestras de plasma de almejas expuestas y no expuestas al parásito en el laboratorio ni entre almejas con infección avanzada y almejas no infectadas tras exposición prolongada en el medio natural. Sin embargo, el número de marcas en los geles del plasma de las almejas del experimento de exposición prolongada (174 marcas por gel de media) fue significativamente menor que el de las almejas del experimento de exposición en laboratorio (389 marcas de media), lo que podría deberse a las diferencias en las condiciones ambientales, con gran influencia en el plasma. Sólo se encontraron marcas exclusivas de tratamiento en los geles del experimento reexposición en laboratorio, con 15 marcas exclusivas del plasma de las almejas no expuestas y 10 marcas exclusivas de las almejas expuestas. Todas esas marcas se procesaron para su secuenciación y posterior identificación en bases de datos; de ellas únicamente se identificaron 14.

Parece que la exposición de las almejas a *P. olseni* en laboratorio indujo en el plasma la expresión de la proteína X1 interactuante con Pin2, la demetilasa 3B especifica de lisina, la proteína de unión de calcio sarcoplasmático y de una lisozima, puesto que dichas proteínas se identificaron en marcas exclusivas de plasma de almejas expuestas. La proteína X1 interactuante con Pin2 está implicada en la regulación de la telomerasa. La demetilasa 3B especifica de lisina provoca cambios en

la estructura de la cromatina y regula procesos celulares de crecimiento y diferenciación. La proteína de unión del calcio sarcoplasmático está implicada en la modulación de la concentración de calcio y su expresión varía en invertebrados infectados. La lisozima es una proteína bactericida cuya expresión en ostras *Crassostrea virginica* aumenta por la infección por *P. marinus*. Por el contrario, la exposición al parásito en el laboratorio pudo inhibir la expresión en el plasma de una nucleótido pirofosforilasa, la proteína 5 de dominio PDZ y LIM, el precursor del péptido del pie 3, la 4-coumarato CoA ligasa 7, caprina-2 y tres lectinas, lactadherina, lectina de reconocimiento del ácido siálico y la ficolina-2, puesto que estas proteínas se identificaron en marcas exclusivas de almejas no expuestas. A excepción de las tres lectinas identificadas, ninguna de las otras 5 proteínas parece estar implicada en procesos inmunes. Las lectinas están implicadas en el reconocimiento y opsonización de patógenos, lo que facilita a los hemocitos reconocerlos y fagocitarlos/encapsularlos.